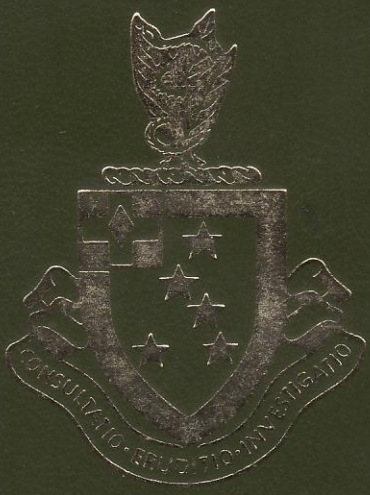
Manual of
HISTOLOGIC STAINING METHODS
of the
Armed Forces Institute of Pathology



Third Edition

American Registry of Pathology

Manual of HISTOLOGIC STAINING **METHODS of the Armed Forces Instituto of Pathology**

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HISTOLOGIC STAINING METHODS

of the

Armed Forces Institute of Pathology

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The Blakiston Division

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Preface

The growth of histotechnology is no better demonstrated than by the growth of this manual which was first compiled by the late Mary Francis Gridley in 1953. The immediate suceess of this manual resulted in a number of mimeographed printings at the Armed Forces Institute of Pathology from 1953 to 1957. The first "published" edition appeared in 1957 and was a revision of Miss Gridley's numerous mimeographed notes by her devoted colleagues at the Armed Forces Institute of Pathology. At the same time her colleagues established a memorial fund in her narre and conceived a plan to perpetuate her most outstanding contribution to histotechnology, "The Laboratory Manual of Special Staining Technics."

Although this edition has been modified to the point that most techniques contained bear little resemblance to those published in past editions, it is only propor to give Miss Gridley credit for the original inception of the manual, especially since it has given histotechnology a tremendous boost and has been an excellent aid to many technicians and pathologists. Credit must be shared also by the late Lawrence P. Ambrogi, Chief, Histopathology Laboratories, and Miss Evelyn F. Ballou who were primarily responsible for the 1957 and 1960 editions. It was their dedication and foresight that made the present edition possible.

In response to the great demand for the manual and the many changes in histological and special staining techniques it became necessary to revise the 1960 edition. Our original intentions were to make moderate revisions, but it soon became apparent that a great deal of revision was necessary if the manual was to reflect all the advances which have taken placo in histotechnology. One need only to leaf through this manual to find that many techniques presented were developed in our Laboratories and have not been published before. Previously published standard techniques have been given new "twists" to simplify and/or better demonstrate the normal structure or pathologic condition.

1 am indebted to Dr. Frank Johnson, who was most helpful with his guidance and suggestions and for review of the entire manuscript; to Miss Evelyn F. Ballou, Mrs. Bertha D. Landi, Mrs. Lillian K. Washington, Mr. David Lewis, Miss Eliza Buddo, Mr. Edward Cunningham, and Mr. Peter Emanuele who modified and contributed some of the techniques. Special thanks are duo Mr. Benedicto Manuel, Mrs. Marcella Grabner, and Mrs. Edna Prophet who spent many hours abstracting, editing and reviewing. I express my gratitude also to my secretary, Mrs. Sula Hughes, for the endless hours she spent in editing and typing the original manuscript, and to the many technicians who co-operated in various ways in preparing this edition.

Acknowledgement is made to Mrs. Lois O. Runyon, Mrs. Ethel Denis, and Mrs. Ruby Irwin who typed the printer's copy. I wish also to extend my

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Chapter 1

Preparation of Tissue

FIXATION

The foundation of all good histological preparations is adequate and complete fixation. Fixation is required to (1) *prevent* post mortem changes such as putrefaction and autolysis; (2) *preserve* various cell constitutents in as life-like manner as possible; (3) *protect* by hardening the naturally soft tissue, thereby allowing easy manipulation during subsequent processing; (4) *convert* the normal semi-fluid consistency of cells to an irreversible semi-solid consistency; (5) *aid* in the visual differentiation of structure by application of biological dyes and chemicals. To accomplish these objectives the tissue should be placed in the fixative immediately upon removal froco the body or as soou after death as possible.

The choice of fixing agent should be determined by the purpose for which the tissue is to be stained or preserved.

Blocks should be cut thin enough so that the fixative will penetrate the tissue within a reasonably short time. To this and the block should not be more than 4mm in thickness and should be immersed in at least teu times its volume of fixative.

Ten percent buffered neutral formalin is the most widely used fixative because it is compatible with most stains. The length of time for fixation depends upon the size of the block and fixative used. It is well to have a clear understanding of the effects of fixation, the time required for complete fixation of specific tissues, and the post fixation handling of tissue specimens.

That many specimens may be ruined, by poor handling subsequent to proper fixation, has been proveu. This generally occurs when one fails to realize that different fixatives require varied times to effect complete fixation; and the specimen may require a particular treatment, immediately following fixation, to insure retention of specific staining properties.

Additional useful knowledge, is the action of a simple fixative on different parts of the tissue specimen. A partial list of the characteristics of certain common simple fixatives and their various effects follows, while more detailed information in this regard can be found in: Baker, J. R.: *Principles of Biological Microtechnique,* New York, John Wiley & Sons, Inc., 1958.

CHARACTERISTICS OF CERTAIN AGENTS USED AS FIXATIVES

FORMALDEHYDE, 10% FORMALIN

REFERENCE. *The Merck Index*, 7th edition, paga 460, 1960, Merck & Company Inc. Rahway, New Jersey.

The formaldehyde solution, approximately 40% formaldehyde gas in water, calted *formalin, is* treated as a 100% solution in making other formalin percent solutions (10 ml formalin and 90 ml H_2O = 10% formalin).

Because it oxidizes to formic acid, it should not have long contact with chromates. In order to counteract the effect of the formic acid, formalin should be *buffered with sodium phosphate, monobasic and dibasic.*

Reactions of the formalin with tissue proteins are numerous and complex, but, it is an accepted fact, that it does not precipitate proteins and only lightly precipitates the other constitutents of the cell.

Formalin neither preserves nor destroys fat, and is good for complex lipids, but has no effect on neutral fats.

Formalin is not a fixative for carbohydrates, it preserves the proteins which in turn traps glycogen so that it is not easily dissolved.

GLACIAL ACETIC ACID

Acetic acid causes the constitutents of the cells to swell, therefore, it is *nevar used alone* but is combined with some other reagent having a shrinking effect.

The penetration is *rapid* and *good.*

The use of acetic acid gives life-like preservation to nuclei and is used when the desire for chromosomes is requested, but it does not aid in staining.

Acetic acid precipitates nucleoproteins, and destroys mitochondria and the Golgi apparatus.

It does not fix lipids or carbohydrates.

It leaves tissue *soft and prevents hardening* by subsequent use of alcohols.

ALCOHOL, ETHYL 70 —100 PERCENT

Absoluta alcohol is used for the preservation of glycogen.

It acts as a reducing agent, being oxidized to acetaldehyde then to acetic acid; for this reason it cannot be combined with chromic acid.

It is slow of *penetration; hardens and shrinks* the tissue in proportion to the percentage used.

It precipitates soluble nucleic acids. It does not forro a compound with protein but abstracts water froco it and *denatures* it, (changes the nature of the protein without adding another atom).

It precipitates all proteins without firmly combining with them. These precipitates become soluble in potassium iodide. Removal of these precipitates (crystals) is necessary before staining.

It facilitates staining with most dyes making the colors more brilliant.

It neither fixes nor destroys carbohydrates.

PICRIC ACID

It is not often used in simple solution, because it causes the cells to shrink markedly. It requires damp storage because of its explosiva nature.

It penetrates well and fixes rapidly.

It precipitates all proteins, forming picrates easily soluble in water. It traps glycogen in protein.

It leaves tissue soft, and, when used in combination, tends to make for easier staining.

POTASSIUM DICHROMATE

It fixes cytoplasm without precipitation.

It is especially valuable in mixtures for the fixation of lipids, particularly phospholipids.

It preserves phosphatides and is used for mitochondria.

Thorough washing is necessary to avoid forming an oxide in alcohol which cannot be removed later.

For conveniente, there are listed hera some of the more commonly used fixatives or fixative mixtures. Ideally, all tissues should be washed in running water after the use of most fixatives before processing, but this is particularly true following the various formalin fixatives.

FORMALIN SALINE SOLUTION

37 — 40% formalin 100.0 M1

Sodium chloride ..... 9.0 gm

Tap water ..... 900.0 ml

A tolerant fixative. Long storage does not create excessiva hardening or damage. When not buffered may cause formation of formalin pigment. It fixes nuclear chromatin in a cliffused homogeneous pattern making it impossible to visualize chromatin distinctly. Therefore, it is not especially useful for routine use. Ideal for the preservation of muco substances.

Formalin with sodium acetate is an excellent fixative and medium in which to store gross blocks of tissue-

FORMALIN-AMMONIUM BROMIDE SOLUTION

37 — 40% formalin, neutralized ..... 15.0 ml

Ammonium bromide ..... 2.0 gm

Distilled water .....85.0 nil.

An excellent fixative for brain tissue on which the silver and gold techniques are to be performed.

FORMALIN ALCOHOL - ACETIC ACID SOLUTION

37 — 40% formalin ..... 10.0 ml

Alcohol, 80% .....90.0 ml

Glacial acetic acid ..... 5.0 ml

Not an ideal fixative for tissue storage and routine use, however, a good agent for rapid fixation of tissue. Prevents solution of carbohydrates before fixation of the protein component is complete. Addition of acetic acid insures fixation of nucleoprotein providing an improved histological picture. Small pieces (2 mm in thickness) usually are completely fixed in *4 - 6* hours.

FORMOL-CALCI UM SOLUTION

Calcium chloride, anhydrous .....1.0 gm

37 — 40% formalin .....10.0 ml

Distilled water .....90.0 ml

This fixative is especially useful when studies on lipids are to be performed.

ZENKER'S SOLUTION

Distilled water .....1000.0 ml

Mercuric chloride ..... 50.0 gm

Potassium dichromate ..... 25.0 gm

Sodium sulfate..... 10.0 gm

Add 5 ml of glacial acetic acid to 95 ml of Zenker's solution before use. (The solution does not keep well after the addition of the acetic acid).

This mixture lias been used since 1894 when Zenker suggested the addition of mercuric chloride to Muller's fluid to improve the fixation of melei. Tissues preserved by this method stain well with many technics, but

5 ml of 37 — 40% formalin just before use). Especially good for demonstrating mitochondria. It fixes well for routine processing.

BOUIN'S SOLUTION

Picric acid, saturated aqueous solution ..... 750.0 ml

37 — 40% formalin ..... 250.0 ml

Glacial acetic acid ..... 50.0 ml

Fix blocks from 4 to 12 hours depending on the size. It is important to wash in, several changes of 50% alcohol for 4 - 6 hours, agitating constantly, to insure proper removal of the picric acid. Store in 70% alcohol. *Note.* The removal of picric acid from tissues is most essential in order to insure proper staining of the tissue sections. It has been demonstrated in our laboratories, that tissues undergo deleterious effects, as evidenced in the staining, when the picric acid has not been properly removed and remains in the tissue throughout the entire processing.

This harmful reaction continues in effect, within the embedded specimen for a number of years. An excellent stained section is secured several days after fixation and conversely, a very poorly stained section is demonstrated several months later upon recutting the same paraffin block. Therefore, the necessity of removing picric acid from tissues cannot be over emphasized.

HELLY'S SOLUTION *See*

Zenker-Formalin, page 4.

CARNOY'S SOLUTION

Absolute alcohol ..... 60.0 ml

Chloroform ..... 30.0 ml

Glacial acetic acid ..... 10.0 ml

One of the best *penetrating and quickly acting* fixatives known. Generally, 3 hours is adequate for normal size tissue. No washing is necessary, and the tissue may be transferred immediately to absolute alcohol. It gives excellent nuclear fixation with preservation of Nissl substances, plasma cells, and glycogen, *but*, it hemolyzes red blood cells.

CLARKE'S SOLUTION

Absolute alcohol ..... 75.0 ml

Glacial acetic acid 25.0

Petroleum-Ether ..... 10.0 ml

Acetone ..... 10.0 ml

Dioxane ..... 10.0 ml

Primarily used for chromosome studies, this fixative preserves the chromatin better than Carnoy's and gives an improved Feulgen reaction. Fixation usually is complete in 12 - 18 hours; small pieces (2mm in thickness) in 2 - 3 hours. Following the use of Newcomer's solution, place in 60% alcohol for 4 hours.

ORTH'S SOLUTION

Potassium dichromate..... 2.5 gm

Sodium sulfate ..... 1.0 gm

Distilled water ..... 100.0 ml
Mix and add: formalin, 37 - 40%..... 10.0 ml

Place in this fixative for 24 hours. Transfer to a 2.5% aqueous solution of potassium dichromate for 48 hours. Wash in running water overnight. Addition of 5 ml of acetic acid improves this fixative for the demonstration of chromaffin cell granules. This fixative does not keep and must be prepared just before use. *Note.* It penetrates fairly rapidly and evenly, but has a tendency to harden tissues. It is recommended for the demonstration of chromaffin cells and glycogen.

GLUTARALDEHYDE

Limited studies in the use of glutaraldehyde as a fixative for electron microscopy and also as a fixative for routine paraffin sections have been conducted. Studies in this area will continue and more precise information, methods, and technics will be evolved. At the present time only limited information concerning glutaraldehyde and its use as a fixative can be obtained. For this reason no specific instructions for fixation are given in this manual. The following referentes on this reagent are recommended:

1. Sabatini, D. D., Bensch, K., and Barrnett, R.: Cytochemistry and electron microscopy: and preservation of cellular ultrastructure of enzymes by aldehyde fixation. *J. Cell. Biol.* 17:19 - 58, 1963

2. Yanoff, M., Zimmerman, L. E., and Fine, B. S.: Glutaraldehyde fixative of whole eyes. *Amer. J. Clin. Path.* 44:167 -171, 1965.

3. Yanoff, M., and Fine, B. S.: Glutaraldehyde fixative of routine surgical eye tissue. *Amer. J. Ophthal.* 63:137 -140, 1967.

PARAFORMALDEHYDE

Paraformaldehyde also has been used recently for the fixation of electron

to assure that the specimen is soft enough to allow cutting with the equipment available. The necessity for decalcification makes it impossible to perform many criticai studies on bone. In some cases, only when special microtomes are available for sectioning undecalcified specimens could the complete histologic picture be made possible.

Bone and other calcified material should be cut finto small pieces (approximately 5mm) with a fine saw before fixation. After adequate fixation, place in a large quantity of decalcifying solution, at least a quart, for blocks of average size. Stirring, agitation, and the use of vacuum hastens decalcification and should be employed when possible. Tissues suspended in **the upper third of the fluid during decalcification will allow the calcium salte to sink to the bottom of the container as they are dissolved. Suspension of tissue can be accomplished in a number of ways: (1) Place tissue in a gauze bag suspended with dental floss or string which has been dipped in hot paraffin; or (2) place in a perforated porcelain dish on the bottom of the container. Since decalcification acide continue to act on tissue specimens during any subsequent tissue handling (including paraffin storage) it is important that every trace of decalcifying solution be removed by washing the specimens in running water for several hours before processing can take place. For determining the decalcification end point methods see page 10. Various methods of decalcification follow:**

PERENYI'S METHOD

1. Place calcified specimen in large quantities of Perenyi's fluid until decalcification is complete.

PERENYI'S FLUID

10% Nitric acid, aqueous.....40.0 ml

Absolute alcohol .....30.0 ml

0.5% chromic acid, aqueous.....30.0 ml

The various ingrediente may be kept in stock, and should be mixed immediately before use. This solution may acquire a blue violet tinge after a short while but this will have no effect on its decalcifying properties.

2. Wash in running water for 2 hours. Store in 95% alcohol if processing cannot continue following this step.

3. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. **Perenyi's fluid is slow for decalcifying hard bone, but is an excellent, fluid for small deposite of calcium, e.g. calcified arteries, coin lesione, and calcified glande. It may also be an excellent decalcifying fluid for avian eye globes which contain bone normally, at the corneal-scleral junction and for human globes which contain calcium due to pathologic conditions. Little hardening effect of tissue takes place, and excellent morphologic detail is preserved with this technique.**

2. Wash in running water for 30 minutes.

3. Neutralize for a minimum of 5 hours in 10% formalin to which an excess of calcium or magnesium carbonate has been added.

4. Wash in running water overnight.

5. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. **This is a fairly rapid method. Care must be exercised, however, to remove specimen from decalcifying solution shortly after decalcification is complete, since overexposure to nitric acid impairs or destroys nuclear staining. Nitric acid is the acid of choice for decalcifying temporal bones. For this procedure see page 48.**

NITRIC ACID METHOD II

1. Place calcified specimen in large quantities of nitric acid-alcohol solution until decalcification is complete. (Change solutions daily for best results).

Alcohol, 80%.....95.0 ml

Nitric acid, concentrated (68 — 70% Sp. gr. 1.41) ..... 5.0 ml

2. After specimens are completely decalcified transfer directly to a 4% aqueous sodium sulphate solution for 3 hours.

3. Wash specimens in running water for 2 hours to remove sodium sulphate. When time permits, overnight washing is preferred.

4. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. **The decalcification effected with this method is considerably faster than that of method I, since readily soluble fats are dissolved somewhat by the alcohol thereby accelerating the decalcifying process.**

FORMIC ACID-SODIUM CITRATE METHOD

1. Place calcified specimen in large quantities of formic acid-sodium citrate solution until decalcification is complete. (Change solutions daily for best results).

SOLUTION A

Sodium citrate ..... 50.0 gm

Distilled water 250.0 ml

1. Place in "decai" solution until decalcification is complete. (Change solution daily for best resulte.)

2. Wash in running water for a minimum of 16 hours, 24 hours preferred.

3. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. **These methods have proveu very useful in our laboratories since by use of any one of them the compounding of solutions Às eliminated. Cellular destruction is very rapid, however, when bone specimens are allowed to remain in, these solutions for several hours after complete decalcification has been achieved. Therefore, it becomes necessary to determine the decalcification end point precisely.**

ELECTROLYTIC METHOD

The electrolytic apparatus is composed of both positive and negative carbon plates for electrodes which are immersed in the solution. The labeled

cation is complete.

DECALCIFYING SOLUTION

Hydrochloric acid, concentrated ..... 80.0 ml

Formic acid, 90% ..... 100.0 ml

Distilled water ..... 1000.0 ml

2. Wash in running water for a minimum of 16 hours, 24 hours preferred.

3. Dehydrate, clear and impregnate with paraffin or process as desired.

CHELATE'S AS DECALCIFYING AGENTS

Organic chelating agents have been recommended for the decalcification of bone specimen. When used, excellent preservation of histologic detail is observed with subsequent demonstration of various tissue componente possible. The slow speed at ,,which these agents work dictates that the size of the specimen for decalcification must not exceed 4mm in thickness.

There is presented below one method which is the most useful in routine histology. For other procedures, however, see the following referentes:

1. Freiman, D. C.: Organic chelating agent in demineralization of bone for histochemical study of alkaline phosphatase. *Amer. J. Clin. Path. 24:* 227 -231, 1954.

2. Trott, J. R.: The presente of glycogen in the rat Tiver following in vitro processing in decalcifying agents. *J. Histochem. Cytochem. 9:* 699 - 702, 1961.

VERSENATE SOLUTION

Versenate ..... 10.0 gm

Distilled water (pH 5.5 to 6.5). ..... 100.0 ml

3. Place in 70% alcohol. Washing is unnecessary.

4. Dehydrate, clear and impregnate with paraffin or process as desired.

REMARKS. **Most bone specimens (3mm thick) are decalcified in *2-4* days but specimens may be left in Versenate solution for as long as *14* days without noticeable effects on the staining quality of the cell. This is especially true if the solution is between 5.5 and 6.5 pH. Versenate may be used for decalcification of dense calcified materiais. This however, may require considerable time; in that event, the Versenate decalcifying solution should be changed at intervals of 3 or *4* days. The advantage of the Versenate method lies in the preservation of the staining affinity of the cells.**

REFERENCE. **Birge, E. A., and Imhoff, C. E.: Versenate as a decalcifying agent for bone. *Amer. T. Clin. Path 22: 192 -193, 1952.***

PROCEDURES FOR DETERMINING END POINT OF DECALCIFICATION

One of the greatest problems encountered in decalcification is the failure of technicians to realize that the selection of a decalcifying fluid is *not* the most important consideration. Most important to be considered, and the one point receiving the least attention, is the removal of calcified specimens from fluids *immediately* after complete decalcification is accomplished. If this is *not* do[p]e, the chances of subsequent good staining reactions are reduced 10% for every 2 hours the tissue remains in the decalcifying solution. It must be recognized that 90% of the poor staining qualities demonstrated in decalcified tissues, is due to this one factor. For this reason, one of the following methods should always be employed in ali laboratories, to determine the decalcification end point.

SPECIMEN FLEXIBILITY METHOD

This method should be used only after considerable experiente with decalcification

Bend specimen gently; if it bends with little resistance it is well decalcified. One may also press the specimen gently with fingernail; areas containing calcium will not --j-i'l't'e (bend inward) and considerable resistance will be noticed.

KERATIN AND CHITIN SOFTENING PROCEDURES

There are no highly satisfactory procedures for softening keratin and/or chitin -'.%-hich would result in both rapid softening and subsequent good section staining. By use of concentrated sulfuric acid with the aid of heat, keratin can be completely dissolved froco the tissue section. However, much tissue destruction also will occur.

For the softening of chitin the following procedure is found to give a satisfactory -estilt.

1. Fix specimens in a fixative of choice.

2. Place specimens in the following solution until completely de-chitinized. Change solution every 2 days for best results.

Mercuric chlori(le	4 0 gm
Chromic acid	0 5 gm
Nitric acid, concentrated	10.0 ml
EtIly1 alcohol, 95%	50.0 ml
Distilled water	200.0 ml

3. Wash in running water for 3 hours.

4. Dehydrate, clear and impregnate with paraffin or process as desired.

Chapter 2

Processing of Tissue

DEHYDRATING, CLEARING, IMPREGNATING, AND EMBEDDING

A specimen brought to the laboratory is usually marked with an identifying number or narre. Keep this identification, with the specimen throughout processing. A11 identifying marks should be made with a soft lead pencil. Do not use ink or wax pencils.

The surface froco which sections are to be cut may be indicated by notching the opposite surface, or by marking it with India ink. An indelible lead pencil may also be used for this purpose. When the tissue is embedded in paraffin, the marked surface of the block is uppermost.

Fixed tissues must be maintained in position by a firm medium so that thin, uni-forni sections, can be cut. Media suitable for this purpose are paraffin, celloidin, and carbowax.

Processing by the paraffin technic is accomplished most rapidly and gives the best results when thin sections of soft tissue are desired. Since paraffin is not miscible with water, the tissue must be dehydrated and then cleared in solutions miscible with paraffin before impregnation.

Well processed tissue is achieved by a step by step infiltration of the required reagents each preparing the tissue for the one to follow, so that the end result will be a section closely resembling the living state of the specimen. Every cell should be recognizable as to type, enabling the pathologist to focus all his attention on the cell pattern which determines the diagnosis. Properly fixed tissue is essential, as the following steps of processing build upon it.

It is always advisable to remove fixatives before processing. For various methods note remarks under each fixative in Chapter 1.

Dehydration is the removal of all extractable water by a dehydrant diffusing through the tissue, and in the process diluting itself 2 — 4%. Some dehydrants used are tetrahydrofuran, acetone, dioxane, isopropyl alcohol, and ethanol.

Alcohol is the most commonly used dehydrant usually tarting *with 80%.* Exceptions to this are when processing tissue with cavities, cysts, and embryos, which will have *less shrinkage and distortion when started in 60%.* Compact or fibrous tissue, such as muscle, brain, lymph nodes, and glands infiltrate more rapidly and completely in the 60%, *especially when vacuum is employed.* Allow sufficient time in, the starting alcohol for complete infiltration of the tissue. The dehydration process continues by upgrading the alcohols to absolute alcohol. Isopropyl alcohol can be used as a substi-tuta for absolute alcohol if necessary, but absolute alcohol is always preferred. *Note* Isopropyl alcohol should not be used to dissolve dyes and

Dioxane is a rapid dehydrant, but the fumes are highly toxic and its use requires careful control in a well ventilated area. Clearing is not a necessary step in dioxane processing and when used the tissue will retain a softer textura than when acetona is used. It is recommended when it is necessary to expose tissues to a dehydrant clearing agent up to 48 hours.

Example: Largo pieces requiring longer exposure for complete clearing and dehydration. *Tetrahydrofuran* possesses essentially the same dehydrating and clearing properties as dioxane.

Clearing reagents must be miscible with the dehydrant and the paraffin. As the dehydrant is removed, the tissue clears, becoming translucent signifying the completion of the process. The one exception is chloroform, since the tissues do not become translucent in it.

Xylene is the most widely used clearing reagem, but it hardens tissue more than chloroform unless the clearing time is controlled. Xylene, toluene, and benzene are difficult to remove during the paraffin impregnation. A11 clear well, however. Cloroform is the clearing agent of choice in our laboratories.

Impregnation is the complete removal of the clearing reagents, by substitution, as the paraffin penetrates the tissue with use of no less than two, and preferably, three paraffin baths. Most laboratories use paraffins with a melting point of 56 — 58 °C. A frequent check of the temperatura of the paraffin baths is a must, since more than 5 OC above melting point of the paraffin will cause excessiva tissue shrinkage and hardening. Vacuum, when applied during embedding, will remove air, gases, and any remaining clearing agent, and at the same time aid in drawing the paraffin finto all areas of the specimen, especially those areas left void by the evacuation of air.

Recommended paraffins for use in embedding are:

Paraplast is perhaps the best embedding medium for use in conventional histological processing. It should not be used, however, when thin-walled circular specimens are to be cut, because it prevents complete expansion of the specimens, such as cross sections of eyes, trachea, cysts, veins, etc.

Bioloid is a good embedding medium in which thin-walled structures can be sectioned satisfactorily. Its elasticity permits its use to give particularly satisfactory results with circular specimens.

Tissuemat possesses somewhat the same properties as *Bioloid. Note.* Paraffins have a tendency to crystallize if exposed for long periods at temperaturas 10 ° above the melting point. This crystallized paraffin makes sectioning difficult and therefore should be avoided. Paraffins may pick up some moisture and small amounts of the clearing agent during processing. However, these can be evaporated by reheating the paraffin for a short time, not more than 30 minutes at 20 ° above the melting point.

EMBEDDING IN PARAFFIN

***Enibedding* is the orientation of tissue in melted paraffin, which when solidified, n---**

cut. Each laboratory has a preferente as to the method used for embedding with the two most often used being lead Us and the Tissue Tech embedding system.

Embedding can be accelerated by the use of a shallow pan which can be purchased or made by any metal worker. This method is used exclusively at Armed Forces Instituto of Pathology for embedding of multiple blocks. Pans with slightly sloping sides, ranging from 1 to 2 inches in depth and 8 x 10 inches in length and width, are satisfactory.

Placed on a masonite rack, which holds it about 6 inches above the desk top, (Fig. 1), the pan is warmed gently with a Bunsen burner, or a 250-watt reflector infrared heat lamp* of 115 -120 volts, having a squeeze holder attached (Fig. 2). This heat lamp may be purchased at most hardware or drug stores. In the pan filled with filtered, melted paraffin, each piece of tissue is placed in position with the appropriate identifying string tag beside it. When all tissues are oriented and in placo, the paraffin is hardened by rubbing an ice cube across, the bottom of the pan. In order to achieve a flat surface for cutting, press specimen down gently with forceps, making lure that all parts of the tissue are flat. When the paraffin

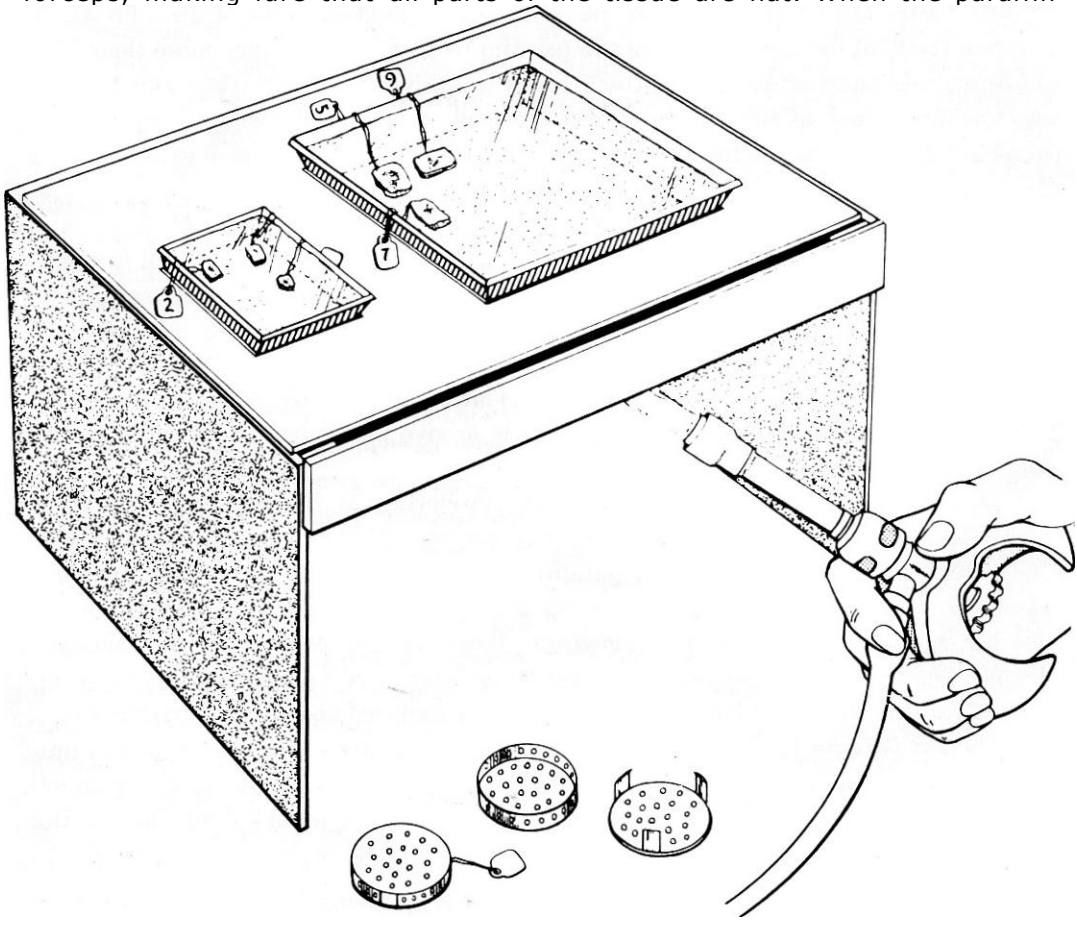


Fig. 1. Multiple block embedding in paraffin.

*Slaughter, E. S., HT(ASCP): The use of the infrared heat lamp for paraffin embedding and blocking. *Amer*

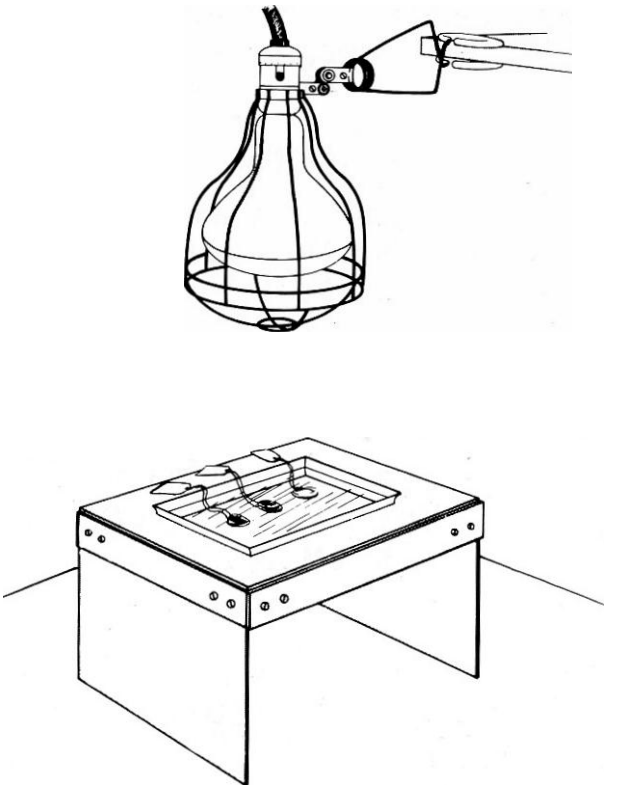


Fig. 2. 250-watt reflector infrared heat Iamp with squeeze holder for suspending larrip above embedding set-up.

GENERAL COMMENTS

When transferring tissue froco impregnating pot to embedding mold, do not allow a thin layer of paraffin to solidfy around the specimen. If by chance it should forro, it must be completely melted to avoid a hair line separation between the tissue and the embedding medium in the blocked specimen. 2. Extra care must be used when embedding multiple pieces of tissue in the same block in matching types and densities of tissue. This simple precaution will facilitate tissue cutting. 3. Imcomplete dehydration will result in poor clearing and impregnation; faulty processing in these areas will produto shrinkage and drying of the specimen when placed in the hot paraffin. This effect can be recognized quickly when a depression forros in the surface of the block, and will also be evident when sectioning, sinto improperly processed tissue turns, white on exposure to water. Sections cut froco a block such as this can be expected to "explode" or disintegrate when placed on the water bath.

e. Improperly processed blocks should be taken back through xylene to remove paraffin, through absoluto alcohol, 95% alcohol and 80% alcohol. Then they should be dehydrated slowly, cleared and reinfiltrated with paraffin and re-embedded. See artifacts pago 245.

available or vice versa. *Note.* Solutions on the tissue processor should be changed once a week when an average of two basket loads of tissue are run each day. The solutions must be kept within one inch of the top of the beaker on the processor.

T

HOLD 1 NG POINT

4:30 P. M. TO
6:30 P. M. TO
7:30 P. M.

8:30 P. M. TO 9:30 P. M.
9:30 P. M. TO 10:30 P. M.

10:30 P. M. TO 11:30 P. M.
11: 30 P. M. TO 12:30 A. M.
12:30 A. M. TO 2:30 A. M.

2:30 A. M. TO

1 M PREGNAT
6:30 A. M. TO

A

Fig. 3. (A)Schedule of processing in paraffin used at Armed Forces Institute of Pathology.

Figure 3B is the processing schedule for the Central Nervous System used at the Armed Forces Institute of Pathology. The use of one of the three methods is dictated by the thickness of the specimen, for example, hand processing may be

WEEK END (2 DAYS)

(Auto Technicon)

	2 HRS	3:00 PM-5: 00 PM	2 HRS	10:00 AM - 12:00 NOON
	1 HR	5:00 PM-6:00 PM	1 HR	12:00 NOON - 2:00 PM
	1 HR	6:00 PM - 7:00 PM	1 HR	2:00 PM - 4:00 PM
ABSOLUTE	1 HR	7:00 PM-4.00 AM	9 HRS	OVERNIGHT
ABSOLUTE	1 HR	4:00 AM - 12 NOON	8 HRS	8:00 AM-9:00 AM
ABSOLUTE	1 HR	12 NOON - 3:00 PM	3 HRS	9:00 AM-10:00 AM
CHLOROFORM	1 HR	3:00 PM - 5:00 PM	2 HRS	10:00 AM-11:30 AM
CHLOROFORM	2 HRS	5:00 PM - 6:00 PM	1 HR	11:30 AM - 1:00 PM
	2 HRS	6:00 PM-7:00 PM	1 HR	1:00 PM 3:00 PM
	2 HRS	7:00 PM -4.00 AM	9 HRS,	3:00 PM 4:00 PM
"PARAFFIN	1 112 HRS	4:00 AM-8:00 AM	4 HRS	SOLI D 1 FY (OVERNIGHT)
*PARAFFIN	2 HRS	8:00 AM-10:00 AM	2 HRS	WARM AND THEN VACUUM FOR 2 HOURS

B

Fig. 3. (B)Processing schedules for routine brain tissue. Normal size not to exceed 5 mm in thickness

OTHER METHODS OF PARAFFIN PROCESSING

The processing time for the following methods are based on specimens which do not exceed 3mm in thickness.

METHOD 1

This method is useful for research and treatment of delicate, as well as hard or densa fibrous tissues, such as skin and uterus, because it has the least hardening effect. Tissues may be left in cedarwood oil for months without any excessive hardening.

Alcohol, 80 1% - 2 changes ..... 1 hour each

Alcohol, 95 /"ó - 2 changes ..... 1 hour each

Alcohol, 100 1% - 3 changes ..... 1 hour each

Alcohol, 100 ɪᴸ⁻'ₒ and xylene - equal parts ..... 1 hour

Oil of cedarwood - 2 changes ..... 2 hours each

Oil of cedarwood -1 change ..... 1 hour

Paraffin -1 change ..... 2 hours

Paraffin - 4 changes ..... 1 hour each

Vacuum the last paraffin change. Embed and cool quickly. Cut as desired.

METHOD II

This method has no specific usefulness except that it is slightly more rapid in penetration of the tissue, due to the penetrative power of benzene — the clearing agent. The advantage of benzene is that the tissue becomes more translucent as the alcohol is replaced, making it possible to determine the tissue clearing and end-point, and thereby avoiding overexposure to the drastic hardening effects of this reagent.

Alcohol, 801"|o - 2 changes ..... 1 hour each

Alcohol, 95%-2 changes ..... 1 hour each

Alcohol, 100% - 3 changes ..... 1 hour each

Benzene - 2 changes ..... 1 hour each

Paraffin-4 changes ..... 1 hour each

Vacuum the last paraffin change. Embed and cool quickly. Cut as desired.

METHOD 111

Vacuum the last paraffin change. Embed and cool quickly. Cut as desired.

METHOD IV

Tetrahydrofuran is being used in some laboratories with good results. It has the same properties as dioxane in that it serves as both a dehydrant and a clearing agent whereby processing schedules can be aecelerated considerably. (We have been able to process thin tissue specimens which do not exceed 3mm in 6 hours). It has also been used very satisfactorily as suggested in advantage 3 of Method III. Tetrahydrofuran is toxic and should be used in a well ventilated area.

Tetrahydrofuran and water - equal parts ..... 1 hour

Tetrahydrofuran, 100% ..... 1 hour

Tetrahydrofuran, 100% ..... 2 hours

Paraffin - 3 changes ..... 1 hour each

Vacuum the last paraffin change. Embed and cool quickly. Cut as desired.

METHOD V

This method is recommended for biopsies as well as bone marrow and cytological buttons. This method for processing minute pieces of tissue prevents the tissue froco becoming hard and brittle due to prolonged periods in the various agents. It is being presented through courtesy of the Walter Reed Army Hospital Pathology Department, Laboratory Service, by Mr. Dan Romeika, Supervisor.

Alcohol, 80% ..... 15 minutes

Alcohol, 95% - 2 changes ..... 15 minutes each

Alcohol, 100% - 2 changes ..... 15 minutes each

Chloroform - 2 changes ..... 15 minutes each

Paraffin - 2 changes ..... 15 minutes each

Embed and cool quickly. Cut as desired.

DOUBLE EMBEDDING METHOD

A double embedding method, in which a combination of celloidin and paraffin is used, may prove advantageous in some instantes. The preservation of the morphological relationship during conventional processing is the usual, and often, the only reason, for employing this technic.

• **celloidin, 2% 24 hours**

Clearing oil mixture ..... 8 hours

CLEARING 011 MIXTURE

Chloroform .....4 parts

Oil of origanum .....2 parts

Oil of cedarwood.....4 parts

Alcohol, 100% 1 part

REMARKS. All contact with xylene and alcohols must be avoided. Carbowax will become brittle if overheated, and crumbling may occur during cutting. The block inust not be allowed to come in contact with ice or water as carbowax is very hygroscopic. If hardening of the block is necessary to eliminate section compression during cutting, it can be hardened with a commercially available freou "Cryokwik"[0], or dry ice. A convenient method used in our laboratories is the placement of a 32 oz funnel filled with dry ice, ovar the specimen, by mearas of a ring stand. The nipple of the funnel is placed 3 -4 inches above the tissue specimen (which is oriented ora 1 he microtome ready to be cut). The cold dry-ice fumes being heavier than room temperatura atmosphere shoot downward, enveloping the specimen. This method keel)s the immediate arca of the block and knife in a considerably colder state facilitating carbowax cutting. Store carbowax embedded blocks in a cool, low moisture arca. For additional information and uses of carbowax the following referentes are recommended, especially, if thin sections (1- 2 mícron) are desired.

REFERENCES. 1. Sidman, R. L., Mottla, P. A., and Feder, N.: Improved polyester wax embedding for histology. *Stain Tech. 36:279 -2 34, 1961.*

Menzies, D. W.: Paraffin-beeswax-stearic acida An embedding mass for thin sections. *Stain Tech. 37: 235 -238, 1962.*

Reid, J. D., and Taylor, D.: An improved method for embedding tissues using polyethlene glycols, with incorporation of low viscosity nitrocellulose. *Amer. J. Clín. Path.* 41: *513-516, 1964.*

°International Equi~nt Co., 300 Second Avenue, Needham Heights, Mass. 38586

Chapter 3

CABE AND USE OF MICROTOME KNIVES

The cutting of good sections depends greatly upon practical experiente and a complete thorough knowledge of the equipment used. Manual (lexterity is a *must;* without it one -may face a difficult task in handling the fine manipulative detail required in section cutting. Hurried and inadequate introductory and/or initial training will reflect badly for years afterward; conversely a high standard of training will prevail admirably throughout one's career. *Speed* in performing any phase of histologic technique should *nevar* be a primary objective lince it only leads to unsatisfactory processing, cutting, and staining of the tissue sections. A well trained tissue technician will produce first rate sections in a far shorter time than one who always is aiming primarily at speed.

Since the results produced by histologic technique depend greatly upon the knives used to cut the sections, it is imperativa that each technician know how to tare for bis knife as well as how to use it. A perfect edge on a microtome knife is difficult to describe, however, with a good knife edge, sections of 3 microns in thickness should easily be cut froco well-processed, average-sized, tissue blocks. The sections should ribbon off the block in a flat unwrinkled fashion, much as papar comes off a printing pres. Microscopically, the section must show no vertical lengthwise scratches or horizontal thick and thin areal. (see Artifacts, paga 247). For problems and possible causes in cutting see Fig. 4.

Knife sharpening can be accomplished by mechanical means on one of several kinds of commercial knife sharpeners. However, in the absence of such assistance, it should not be difficult to acquire the skill and ability to keep one's knife in a satisfactory condition using the hand-honing method. The quality of the section produced, more than compensates for the time spent, in learning to keep, and in keeping, a knife properly sharpened.

In hand-honing, naturally, good quality stones give the best results. They are expensive, but only the best should be used. The finer the grain in a hone the harder the stone. The yellow Belgian and the Belgian black vein are the finest available anywhere and are, therefore, highly recommended. It is best to purchase a combination hone: Belgian yellow vein and Belgian black vein, mounted back to back.* A liquid medium for sharpening with a hone is necessary, such as household 3 in 1 oil, mineral oil, vegetable oil, or a neutral soap solution. The choice must be left to the technician. At the Armed Forces Instituto of Pathology, the neutral soap solution is used because it can be made readily by dissolving household (bar) soap in water.

While honing, the knife should be kept flat, held to the hone by its own weight, with its edge facing the direction of the "heel to toe" motion, under continuous but light pressure (Fig. 5).

***Microtome Knife Hone-Yellow Belgian, Fisher Scientific Co., 7722 Fenton St., Silvar Spring, Md. 20910**

	CRO
	KNIFE AND BLOCK NOT PARALLEL. BLOCKS NOT SQUARE OR RECTANGULAR. IRREGULAR KNIFE EDGE
	COMPRESSED,WRINKLED OR JAMMED SECTIONS
	DULL KNIFE, KNIFE ANDIOR BLOCK WARK TOO VERTI CAL KNIFE TILT.
	CRUMB LING
	INCOMPLETE DEHYDRATION, CLEARING ANDIOR INFILTRATION. PARAFFIN TOO HOT DURING INFILTRATION ANDIOR
	SPLIT RIBBONS
	NICKS IN KNIFE. DIRT ON BLOCK OR KNIFE, KNIFE TILT TOO GREAT.
	LIFTING OF SECTIONS OR RIBBON ON UPSTROKE
	TOO VERTICAL A KNI FE TILT. D I RTY KNIFE EDGE. DULL KNIFE,
	TH IC
	TOO LARGE A BLOCK. LOOSE SET SCREWS FOR BLOCK ANDIOR LOOSE KNIFE HOLDER. BLOCK TOO HARD TO SECTION WITHOUT SOAKING.

PREPARED BY: MRS. E. B. PROPHET, AFIP, HISTO LABS

Fig. 4. Commonly encountered problems in cutting and their causes.

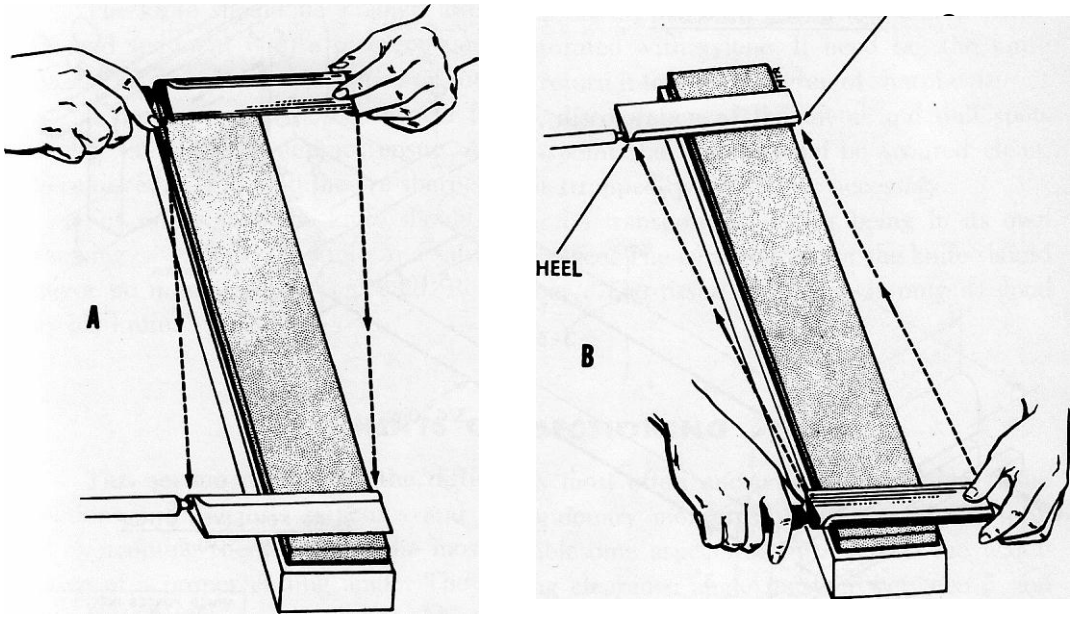


Fig. S. Diagram of the technique of honing the microtome knife.

or linen strop (Fig. 6) to remove the microscopic knife serrations caused by any one of the various sharpening methods.

This important step is often *minimized,* and is responsible for many cutting problems. Regardless of the stropping device used, stropping should be performed so that ali arcas of the knife edge are exposed to the strop surface, equally if possible. The movement of the knife should be from toe to heel for 8 to 12 times *(Fig. 7A).* This movement should then be reversed to from heel to toe 8 to 12 times (Fig. *7B).*

However, it must be clearly understood that it is possible to ruiu a well-sharpened knife during the stropping phase. The most common defect noted is a turned or rounded edge, which happens when too much pressure is applied to the knife while stropping or as in the case of the linen strop, when the linen is not stretched tautly enough. Theoretically, the cutting edge is not a true edge. It is, in fact, a tiny part of a circumference of a minute circle with the arc measuring approximately *0.25* microns. The length of this arc is increased considerably in a rounded edge, sometimes to as much as *0.75* microns.

This becomes important if one remembers the theoretical mechanism by which sections are cut. The impregnating matriz and tissue in the block are compressed in front of the cutting edge of the knife, which in turn wedges off the section in a tearing crushing manner at submicroscopic leveis. It stands to reason, then, that the wider the circumference of the arc the more crushing and tearing effect will prevail and more resistance will be met by the knife edge. This resistance will always result in considerable compression of a section — a sign of a poorly sharpened knife (see

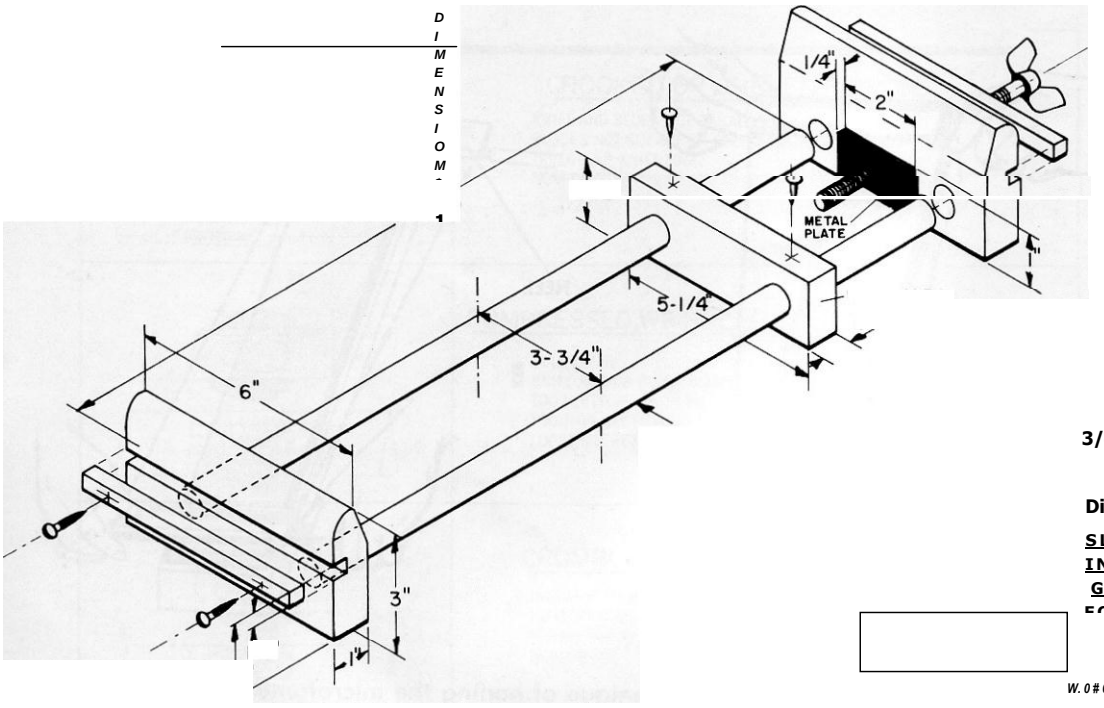
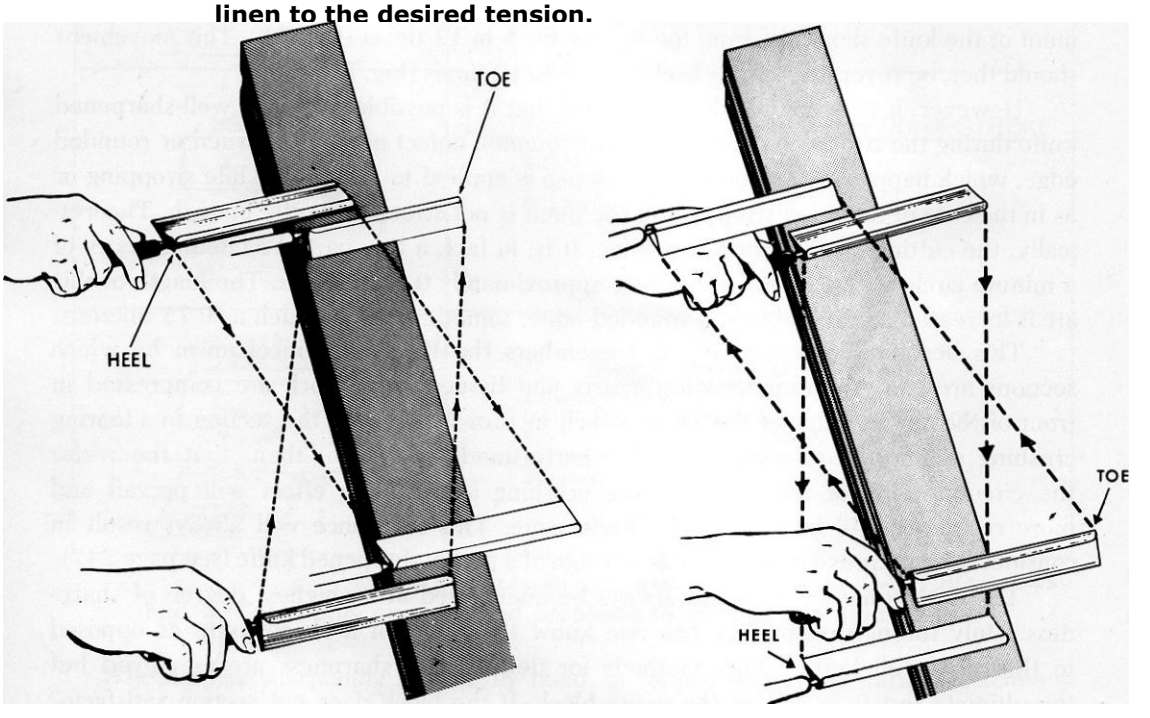


Fig. 6. Sling for a linen strop. Linen for the strop can be purchased at most fabris stores. It should be cut to the length and width dictated by the sling with pinking shears and the linen attached to the sling with the wooden slats and screws shown in the drawing. The large screw at the head of the sling facilitates stretching the linen to the desired tension.



A

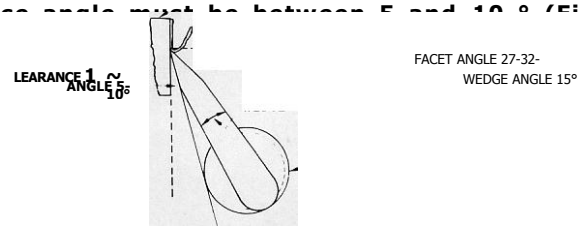
- **The knife should be cleaned after each use by removing the accumulated paraffin and sediment with a piece of gauze saturated with xylene. If need be, the knife should be sharpened or stropped or both, to return it to a high degree of sharpness.**

If the knife is exposed to acid fumes, discoloration of the metal and dull spots on the knife w111 sometimes ensue. At this point the knife should be scoured clean, to remove the acid, and then re-sharpened or stropped, whichever is necessary.

For safety sake the knife should never be transported without being in its own carrying case. Store the knife in a safe, cool place. The tare and use of the knife should never be minimized or neglected. Remember, "The tissue technician is only as good as his knife."

HINTS ON SECTIONING

This section deals with the difficulties most often encountered in cutting tissue sections and suggests remedies and precautionary measures which may be helpful in overcoming them. One of the most troublesome aspects of cutting is in the acquisition of a proper cutting angle. The cutting clearance angle must be between 5 and 10 ° (Fig. 8)



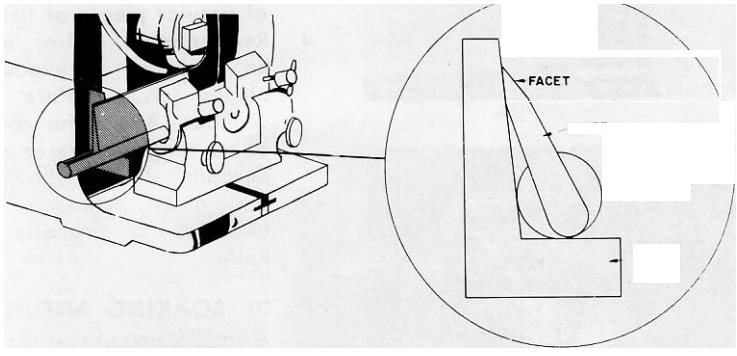


Fig. 8. Diagram showing the use of an angle **jig to assure the proper cutting** angle.

An angle jig is simple to use: Place the knife in the microtome knife clame making certain it protrudes at least four inches to one side. Place the knife back on the protruding end of the knife. The angle jig is then placed behind the

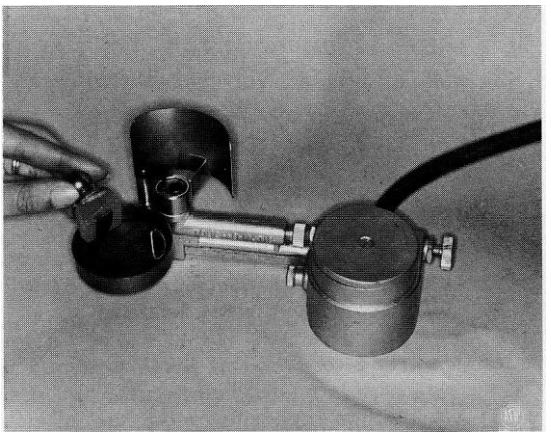


Fig.14. RESEALING BLOCKS

1. **After the desireci number of sections are taken ali blocks should be paraffin sealed. This prevents drying and other damage which can alter and sometimes ruiu the tissue, making future sectioning difficult or impossible.**

ATTACHING SECTIONS TO SLIDES (SECTION ADHESIVES)

[p]araffin sections may be attached to slides in several ways. A sma11 drop of Mayer's egg albumin is smeared over the surface of the slide with the finger and the excess rubbed off with the heel of the hand, or it can be applied with a clean foam-rubber sponge. A sponge is usually preferred so that the epithelial cells froco the finger wi11 not adhere to the slide and produce artifacts when slides are stained.

MAYER'S EGG ALBUMIN

Egg white .....50.0 ml

Glycerin .....50.0 ml

Mix we11 and filter through coarse filter paper or Buchner's funnel with vacuum or through several thicknesses of gauze, add a crystal of thymol to preserve. Commercial egg albumin can be purchased, ready for use.

ADHESIVE FROM DRIED ALBUMIN

Albumin, dried .....5.0 gm

Sodium chIonde ..... .0.5 gm

Distilled water.....100.0 ml

Filter through Buchner's funnel with vacuum, to 50 ml of filtrate, add 50 ml of glycerin. Add a crystal of thymol to preserve.

GELATIN ADHESIVE

- **Dissolve with the aid of heat. Add several crystals of thymol to preserve. For use, thoroughly mix 3 teaspoonfuls of the 5% gelatin solution per 1000 ml, in the flotation bath.**

Although the section adhesives presented above have proven to be most satisfactory for routine sections, there frequently crises the need for a more firm section attachment. For example, when sections are treated with potassium permanganate, enzymes, or extremely alkaline solutions, they have a tendency to become separated from the slide. The following methods have proven useful in providing a more firm section attachment in such instances.

GELATIN-FORMALIN

Formalin hardens the protein contained in gelatin and egg albumin. This effect can be accomplished by exposing gelatinized or albuminized slides to formalin and thus achieving a firmer bond between the section and the glass slide.

1. Gelatin or egg albumin is applied as described earlier.
2. Sections are placed on slides from the flotation bath and drained from *30* seconds to 1 minute.
3. Placo slides in a coplin jar containing approximately 5 ml of formalin (full strength), making certain the formalin solution in no way comes in contact with the tissue section. The tightly covered coplin jar is then placed in a 60 ' C oven for one hour.
4. Remove sections from coplin jar and dry in the conventional way i.e., either on slide warmer overnight or in an oven for several hours.
5. Treat and/or stam slides as desired.

THIN CELLOIDIN ADHESIVE FOR PARAFFIN SECTIONS

Paraffin sections which tend to become separated from the slide because of the action of acids and alkalis used in the various staining procedures, and which are extremely dried or which contain bone and keratinized material may be held more firmly to the slide if they are coated with a thin film of 0.5 — 1% celloidin before staining.

1. Deparaffinize the slide in xylene and run through several changes of absolute alcohol.
2. Immerse in 0.5 — 1 % celloidin solution for 1 to *2* minutes.
3. Air-dry sections for *30* minutes or until sections turn white.
4. **Immerse sections in *80%* alcohol for 1 to *2* minutes.**
5. Rinse in distilled water and proceed with desired stain.

H. C. Cook conducted a study on section

ıe Hiato-Freeze" and/or Section-Freezet are excellent units for cutting frozen sections. Each instrument consista of a portable box chamber which generates pressurized liquid freon. These units have become very popular since they obviate the need for carbon dioxide tanks which were necessary when using the clinicai micro-tomes. At the Armed Forces Instituto of Pathology the sliding microtome is found to be the most useful. Still another piece of equipment for producing frozen sections is the well known Cryostat. Regardless of the equipment used frozen section procedures must not be taken lightly and every effort made to achieve the best possible resulta in both cutting and staining.

The following technic is preferred in our laboratory:

1. Fix small blocks of tissue in. 10% buffered neutral. formalin.

2. Remove fixative by washing specimens in water before cutting.

3. Placo a drop of water on the specimen holder and place the block in position parallel to the knife edge.

4. Apply gentio downward pressure on the tissue with a glass slide and release freon until specimen is frozen. (Directions for use of the freon units are provided by the manufacturer, therefore, they will not be repeated hera).

ırrently there are available embedding compounds which will assist in cutting, and are, therefore, recommended when frozen sectioning. These, embedding matrix M-1 t or O. C. T. (Optimum cutting temperaturas) * compound provido a surrounding matrix which holds the block and prevents shattering at the free end. It is also possible to impregnate tissue blocks with this material. (Directions for their use are provided by the manufacturer).

5. Start sectioning and continue until a complete section is obtained. If specimen is frozen too hard the section will shatter. In this case thaw the surface of the block by placing a finger on the specimen, but if it becomes too soft sections w111 be impossible to obtain. The correct temperatura can only be judged by experiente. It is best to cut slowly.

6. Lift the section from the knife with the middle finger which has been dipped in distilled water and place the section in a dish of distilled water (wipe the knife free of water between sections since water will cause the sueceeding section to be uneven or perforated). Sections may be picked up on albuminized slides and dried before staining or they may be stained singly by carrying each section through the various solutions with a bent glass rod. A simple aid in staining frozen sections in quantity is the sieve-like container made from 1 inch sections of plastic or glass tubing with walls 1/8 inch thick and the diameter ranging from 1- 3 inches. Ovar one and of each ring a piece of nylon stocking is stretched tightly and glued to the outside wall with Duco Coment. When the cement is thoroughly dried the excess nylon is trimmed off. These containers are transferred from one solution to another with forceps.

REFERENCE **Albercht, M.** ***H : Stain Tech. 31: 231, 1956***

GELATIN SOLUTIONS, 5, 10, & 20%

Gelatin solutions of pure pharmaceutical grade gelatin are prepared in strengths of 5, 10, and 20 percent. If made in quantity, add a few crystals of thymol as a preservativa.

1. Wash specimens thoroughly in running water.

2. **Impregnate with 5% gelatin in 40 ° C oven for 16 hours.**

3. Continue impregnation with 10% gelatin in 40 ° C oven for 16 hours. *Note.* If vacuum is used the time in steps two and three may be cut to 2 hours each.

4. Embed in 20% gelatin.

5. Placa in refrigerator overnight.

6. Trim block for sectioning.

7. **Harden by immersion in 10% formalin (the length of time in formalin to be determined by the desired firmness of the block).**

0

Progressive staining is accomplished by employing a hematoxylin solution which contains an excess of aluminum salte or acid, thus increasing the selectivity for nuclei. After staining with hematoxylin, the slides are wisheci'well in water and the secondary stain applied.

In *regressiva* staining, the sections are overstained in a relatively neutral solution of hematoxylin. The excess stain is removed with an acid alcohol solution, then the sections are neutralized with an alkaline solution such as weak ammonia water or lithium carbonate water. This differentiation is controlled by the use of the microscope, and with experience and a trained eye, the technician can produce excellent resulte. In a well-differentiated section, the cytoplasm should be colorless and nuclear substances should be clearly visible, showing some metachromatic properties.

Counterstains for hematoxylins are a matter of personal preference, with eosin probably the one most commonly used. Too much time will not be devoted to the discussion of Counterstains; but there are a couple of suggestions which should be marfe. Counterstains are defined as stains applied to render the effects of miother stain more discernible. Although this is true, m many cases, it does not apply to the H&E techmque lince, in this case, it plays a definite diagnostic role iii its demonstration of inclusimi bodies and cytoplasmic changes. More correedy, it should be coiisidered a secondary stain. With this in mind, one should realize the value of the various steps subsequent to eosin staining.

The most common error often encountered is the improper use of alcohóls after lhe eosin has been applied. The primary purpose of the alcohols is deliydratimi. But Just as importam is the removal of excess eosin. By passing rapidly through these alcohol solutions excess eosin will overshadow many diagnostic features, such as pigmente and inclusion bodies. It cannot be stressed enough that proper differentiation of the eosin by the alcohol is a *must,* in order to produce 11&E sections of high quality. Therefore, it follows that proper differentiation of eosin, or any couriterstaiii, should be considered just as importam as proper differentiation of hematoxylin.

REMARKS. Poor hematoxylin staining can often be attributed to improper mixing of the aluminum ammonium sulfate or aluminum potassium sulfate. These chemicals serve as mordants for the hematoxylin and therefore must be in complete solution with the other ingredients. The directions given for mixing hematoxylin should be 1'ollowed closely. *When mixing hematoxylins be certairi that ali ingredients are in soluII*Mi.* For artifacts produced in H&E staining see pago 250.

The following is a partial list, with directions for compouríding, of the more frequently used hematoxylins.

MAYER'S HEMATOXYLIN

Hematoxylin crystals	1.0 gm
Distilled water	1000.0 ml
Sodium iodate	0.2 gm
Ammonium or potassium alum	50.0 gm
Citric acid	1.0 gm
Chloral hydrate	50.0 gm

Dissolve the alum in water, without heat; add and dissolve the

HARRIS' HEMATOXYLIN

Hematoxylin crystals ..... 5.0 gm
Alcohol, 100% ..... 50.0 ml
Ammonium or potassium alum ..... 100.0 gm
Distified, water ..... 1000.0 MI,
Mercuric oxide (red) ..... 2. ˜a gin

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as *rapidly* as possible. (Limit this heat to less than 1 minute and stir often). Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel finto a basin of cold water until cool. The stain is ready for use as soou as it cools. Addition of 2 - 4 ml of glacial acetic acid per 100 ml of solution increases the precision of the nuclear stain. Filter before use.

DELAFIELD'S HEMATOXYLIN

Hematoxylin crystals ..... 8.0 gm
Alcohol, 95% ..... 50.0 ml

Ammonium or potassium alum, saturated aqueous solution

(approx. 15 gm/100 ml) ..... 800.0 ml

Add the hematoxylin dissolved in the alcohol to the alum solution and expose to the light and air in an unstoppered bottle for 3 -5 days. Filter and add:

Glycerin ..... 200.0 ml
Alcohol, 95% ..... 200.0 ml

Allow the solution to stand in the light approximately 3 days filter and keep in a tightly stoppered bottle.

BOHMER'S HEMATOXYLIN

Hematoxylin crystals ..... 5.0 gm
Ammonium or potassium alum ..... 100.0 gm
Alcohol , 80% ..... 60.0 ml
Distilled water ..... 1000.0 ml

Dissolve the hematoxylin in the alcohol and the alum in distilled water. Mix and ripen by exposure to light and air for about 2 weeks.

BULLARD'S HEMATOXYLIN

Hematoxylin crystals ..... 8.0 gm
Alcohol, 80% ..... 144.0 ml
Glacial acetic acid ..... 16.0 ml

Dissolve by gentle heat and add mixture of:

Ammonium ox potassium alum ..... 20.0 gm
Distilled water ..... 250.0 ml

Heat to boiling and then remové froco flame and add slowly:

Mercuric oxide ..... 8.0 gm

Cool quickly, filter and, add mixture of:

Alcohol, 95% ..... 275.0 ml

Glycerin .....330.0 ml
Glacial acetic acid..... 18.0 ml
Aimuomwn or potassium aliiiii ..... 40.0 gm,

EHRLICH'S HEMATOXYLIN

Hematoxylin crystals ..... 4.0 gm
Alcohol, 95% .....200.0 ml
Ammonium or potassium alum..... 6.0 gm
Distilled water .....200.0 ml
Glycerin .....200.0 ml
Glacial acetic acid ..... 20.0 ml

Dissolve the hematoxylin in the alcohol and the alum in distilled water and mix. After these are in complete solution add the glycerin and acetic acid.

Note: Aluminum ammonium sulfate is known as ammonium alum. Aluminum potassium sulfate is known as potassium alum.

WEIGERT'S IRON HEMATOXYLIN

Solution A

Hematoxylin crystals ..... 1.0 gm
Alcohol, 95% .....100.0 ml

Solution B

Ferric Chloride, 29% aqueous.....4.0 ml
Distilled water .....95.0 ml
Hydrochloric acid, concentrated ..... 1.0 ml

Working Solution

Equal parts of Solution A and Solution B.

Not recommended for routine use. A very good and useful nuclear stain for specíal stains requiring hematoxylin.

Additional information and the formulae for over 60 hematoxylins can be found in: Gray, P.: The Microtomist's Formulary and Guide, New York, McGraw-Hill Book Co., 1954.

COUNTERSTAINS FOR HEMATOXYLIN

Many counterstains could be listed here which would give good and almost identical, results. It is for this reason that we will only list those counterstains which give different staining results.

1% STOCK ALCOHOLIC EOSIN

Eosin Y, water soluble .....1.0 gm
Distilled water..... 20.0 ml

Dissolve and add:

Alcohol, 95% 80.0

WORKING EOSIN SOLUTION

Eosin stock solution ..... 1 part
Alcohol, 80% ..... 3 parts

Just bafore use and add 0.5 ml of glacial acetic acid to each 100 ml of stain and stir.

EOSIN-PHLOXINE SOLUTION

Stock Eosin

Eosin Y, water soluble ..... 1.0 gm
Distilled water ..... 100.0 ml

Stock Phloxine

Phloxine B ..... 1.0 gm
Distilled water ..... 100.0 ml

Working Solution

Stock Eosin ..... 100.0 ml
Stock Phloxine ..... 10.0 ml
Alcohol, 95% ..... 780.0 ml
Glacial acetic acid ..... 4.0 ml

Make up working solution as needed. Working solution should be changed at least once a week.

PHLOXINE AND SAFRAN SOLUTION

These solutions have been used collectively or individually as counterstains for hematoxylin. For these procedures see routine staining methods paga 39.

METHOD 1

ROUTINE MAYER'S HEMATOXYLIN AND EOSIN STAIN

(Progressiva Stain)

Mayer's hematoxylin is used because it eliminates the necessity for differentiation and bluing of the section. It can be considered a progressiva stain which produces a stained section with a clearly defined nuclei while the background is completely colorless. The biggest objection to Mayer's hematoxylin as used in the past, has been that stained slides often fade after 1 to 3 years. This problem can be eliminated, however, when the slides are washed, after the hematoxylin, in running water for a minimum of 20 minutes.

This method givés consistent results even when more than one person stains sections froco the same block. Also, slides may be left in the hematoxylin for hours without overstaining. Because of the simplicity of the technique, it is possible to teach others to use it within a shorter time as well as a definita reduction in time performance of

the stain itself. See Figure 15 for the steps and timing with use of Mayer's Hematoxylin and Eosin procedure.

F 1 XAT 10 N. Any well fixed tissue.

TECHNIQUE. Paraffin, celloidin, or frozen.

SOLUTIONS

MAYER'S HEMATOXYLIN

(See page 33)

EOSIN SOLUTIONS

(See page 35)

GRAMS OR LUGOL'S **IODINE**

(See page 41)

STAINING **PROCEDURE**

1. Deparaffinize and hydrate to water.
2. If sections are Zenker-fixed, remove the mercuric chloride crystals with iodine Iw and clear with sodium thiosulphate (hypo) (soe page 41).
3. Mayer's hematoxylin for 15 minutes.
4. Wash in running tap water for 20 minutes.
5. Counterstain with eosin froco 15 seconds to 2 minutes depending on the age of the eosin, and the depth of the counterstain desired. For even staining resulte dip slides several times before allowing them to set in the eosin for the desired time.
6. Dehydrate in 95% and absoluto alcohols, two changes of 2 minutes each or until excess eosin is removed. Check under microscope.
7. Clear in xylene, two changes of 2 minutes each.
8. Mount in Permount or Histoclad.

RESULTS

Nuclei - blue - with some metachromasia.

Cytoplasm - various shades of pink - identifying different tissue componente.

REMARKS. The adhesives used to attach sections onto the slides (gelatin, egg albumen) will *sometimes* stain, in areas around the section, with Mayer's hematoxylin. This '.%-W give the slides a slightly dark appearance but in no way affects the nuclear staining. To remedy this, use 10 -12% glacial acetic acid in 95% alcohol, to "clean" the slides after Mayer's hematoxylin. Following with a few dips in saturated aqueous lithium carbonate, the nuclei will blue immediately. This is optional, for the 20-minute wash m running water is sufficient to blue the nuclei. This step will in *no way alter* or *minimize* the staining of the nuclei.

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, o55 - ashington, D.C. 20305.

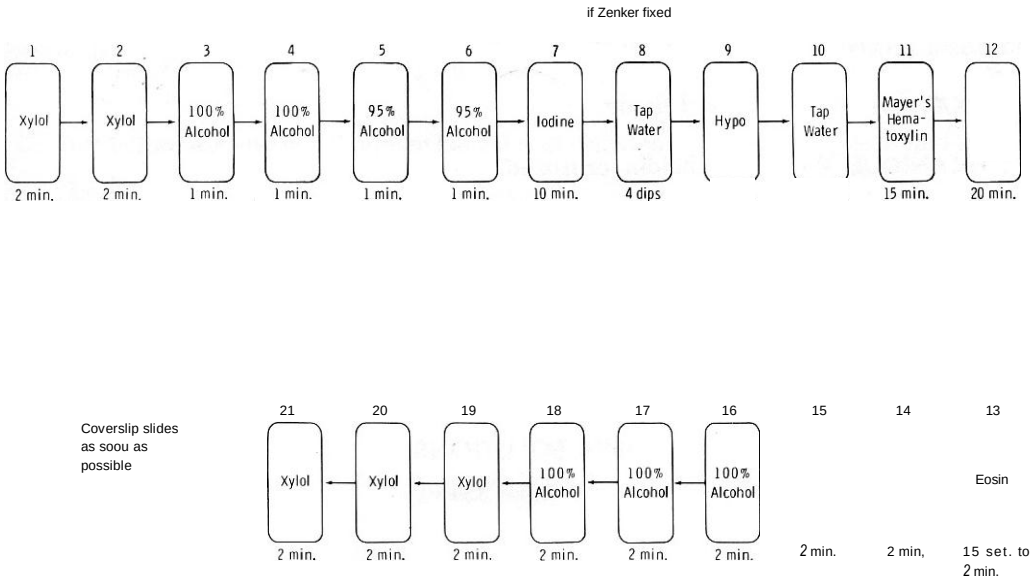

if Zenker fixed
1 Xylol 2 min.
2 Xylol 2 min.
3 100% Alcohol 1 min.
4 100% Alcohol 1 min.
5 95% Alcohol 1 min.
6 95% Alcohol 1 min.
7 Iodine 10 min.
8 Tap Water 4 dips
9 Hypo
10 Tap Water
11 Mayer's Hema-toxylin 15 min.
12 20 min.
Coverslip slides as soou as possible
21 Xylol 2 min.
20 Xylol 2 min.
19 Xylol 2 min.
18 100% Alcohol 2 min.
17 100% Alcohol 2 min.
16 100% Alcohol 2 min.
15 2 min.
14 2 min,
13 Eosin 15 set. to 2 min.

Fig. 15. Steps in staining sections with Mayer's hematoxylin and eosin.

METHOD II

ROUTINE HARRIS HEMATOXYLIN AND EOSIN STAIN

(Regressive Stain)

This is also an excellent staining procedure for the routine hematoxylin and eosin stained slides.

FIXATION. May be used after any fixation.

TECHNIQUE. Paraffin, celloidin, or frozen.

SOLUTIONS

HARRIS' HEMATOXYLIN

(See page 34)

ACID ALCOHOL

Alcohol, 70% ..... 1000.0 ml
Hydrochloric acid, concentrated ..... 10.0 ml

AMMONIA WATER

Tap water ..... 1000.0 ml
Ammonium hydroxide, 28% ..... 2-3 ml

SATURATED LITHIUM CARBONATE

Lithium carbonate 10 gm
Distilled water ..... 100.0 ml

EOSIN SOLUTIONS

(See page 35)

STAINING PROCEDURE

1. Deparaffinize and hydrate to water.
2. If sections are Zenker-fixed, remove the mercuric chloride crystals with iodine and clear with sodium thiosulphate (hypo) (see page 41).
3. Harris' hematoxylin for 15 minutes.
4. Rinse in tap water.
5. Differentiate in acid alcohol, three to ten quick dips. Check the differentiation with a microscope. Nuclei should be distinct and the background very light or colorless.
6. Wash in tap water very briefly.
7. Dip in ammonia water or lithium carbonate water until sections are bright blue, (three to five dips).
8. Wash in running tap water for 10 to 20 minutes. If washing is inadequate eosin not stain evenly.
9. Stain with eosin for 15 seconds to 2 minutes depending on the age of the eosin and the depth of the counterstain desired. For even staining results dip slides sororal times before allowing them to set in the eosin for the desired time.
10. Dehydrate in 95% and absoluto alcohols until excess eosin is removed, two changes of 2 minutes each. Check andor microscope.
11. Absoluto alcohol, two changes of 3 minutes each.
12. Xylene, two changes of 2 minutes each.
13. Motim in Permount or Histoclad.

RESULTS

Nuclei	blue - with some metachromasia.
Cytoplasm	various shades of pink-identifying different tissue componente.

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D. C. 20305.

METHOD 111

ROUTINE HEMATOXYLIN-PHLOXINE-SAFRAN STAIN

This method is often requested in our laboratories for color differentiation of various tissue componente (see results), and for that reason may be valuable to other laboratories. FIXATION. May be used after any fixative.

TECHNIQUE. Paraffin, or frozen sections (we have not used this method for celloidin).

MAYER'S HEMATOXYLIN

1.5% AQUEOUS PHLOXINE B SOLUTION

Phloxine B ..... 1.5 gm
Distilled water ..... 100.0 ml

2.0% ALCOHOLIC SAFRAN*

Safran du Gatinais ..... 2.0 gm
Alcohol, 100% ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to water.
2. Placo in saturated aqueous picric acid for 5 minutes.
3. Rinse in tap water until all the picric acid is removed.
4. Stam in Mayer's hematoxylin for 15 minutes.
5. Wash in running tap water for 20 minutes.
6. Stain in 1.5% aqueous solution of phloxine B for 2 minutes.
7. Wash in tap water for 5 minutes.
8. Dehydrate in three changes of absolute alcohol.
9. Stain in 2% alcoholic Safran for 5 minutes.
10. Rinse with two changes of absolute alcohol.
11. Xylene, two changes of 2 minutes each.
12. Mount in Permount or Histoclad.

RESULTS

Nuclei blue
Red cells vermillion pink
Bone -yellow
Cartilage - yellowish green
Muscle -red
Collagen - yellow

The various effects possible with different fixation are too numerous to be reported here. Only by the use of this technique can these effects be appreciated.

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D. C. 20305.

METHOD]V

ROUTINE METHOD FOR STAINING "RUSH" FROZEN SECTIONS

This method is provided by the Pathology Department, Laboratory Service, Walter Reed Army Hospital. This is the method of choice in that laboratory for staining rush frozen cut sections.

FIXATION. Tissue may be fixed in 10% formalin with the aid of heat or cut unfixed on a cryostat.

TECHNIQUE. Cut frozen section and float on water.

STAINING PROCEDURE

1. Float section onto albuminized slide and blot gently but completely.
2. ..... Alcohol, 100% ..... 1 dip

*Safran du Gatinais, Roboz Surgical Instrument Co., 810 - 18th Street, N.W., Washington, D.C. 20006.

3. Acid alcohol ..... 1 dip
4. ..... Water 1 dip
5. Hematoxylin ..... 1 min. (Place hematoxylin on slide with dropper, hold slide near slide warmer.)
6. Warm water ..... 1 dip
7. Acid alcohol ..... 1 dip
8. Water ..... 1 dip
9. Sodium carbonate, saturated aqueous ..... 1 dip
10. Water ..... 1 dip
11. Alcohol, 80% ..... 1 dip
12- Eosin ..... 1 dip
13. ..... Alcohol, 95% 1 dip
14. ..... Alcohol, 100% 1 dip
15. ..... Alcohol, 100% 1 dip
16. ..... Carbol-xylene 1 dip
17. ..... Xylene 1 dip
18. ..... Xylene 1 dip
19. Mount with Permount or Histoclad.

RESULTS

Nuclei - blue
Cytoplasm - pink

qw REFERENCE. Pathology Department, Laboratory Service, Walter Reed Army Hospital, Washington, D. C. 20305.

REMOVAL OF PIGMENTS AND PRECIPITAM

Our presentation of pigment removal methods will be limited to those specimens containing formalin, mercury, melanin and malaria pigment.

For an excellent review of histochemical methods for the demonstration of pigments see Thompson, S. W.: *Selected Histochemical and His topa thologica 1 Method.* Springfield, Charles C Thomas, 1966.

MERCURY PRECIPITAM PIGMENT

GRAM'S IODINE

Iodine 10 gm
o Potassium iodide ..... 2.0 gm
Distilled water ..... 300.0 ml

LUGOL'S IODINE

Iodine ... . 1.0 gm
Potassium iodide ..... 2.0 gm
Distilled water..... 100.0 ml

3. Rinse in tap water.
4. Place in 5% aqueous sodium thiosulfate (hypo) for 3 minutes.
5. Wash in tap water for 10 minutes or longer. Stain as desired.

MELANIN PIGMENT

There are a number of pigments referred to as melanin in both normal and abnormal conditions. These pigments may appear to be closely related, but they are probably different in chemical structure. Regardless of this possibility, we find the use of potassium permanganate a very satisfactory chemical for the removal of melanin pigment.

One must keep in mind however, that this procedure may alter considerably the staining properties sinto potassium permanganate is very harsh on the tissue section. To prevent loss of sections during staining it may be necessary to coat them with thin celloidin (see page 29).

Alternatively, one can use the gelatin-formalin procedure suggested on page 29.

METHOD 1

1. Deparaffinize and hydrate to water.
2. Rince in distilled water.
3. Place in 0.25% aqueous potassium permangenate solution for 30 minutes to 1 hour (time to be determined by the amount of pigment prosem).
4. Wash well in water.
5. Place in 5% aqueous oxalic acid solution or hydrobromic acid solution (1 part, 3 parts distilled water) until sections become clear (2-5 minutes).
6. Wash in tap water for 10 minutes. Rinse in distilled water and stain as desired.

METHOD II*

This method has proveu to be of value in removing melanin froco friable or difficult tissues. Especially useful when one experientes difficulty in' retaining sections on slides because of the harsh effect of the potassium permanganate.

1. Cut paraffin or frozen sections.
2. Float sections on a conventional flotation bath to allow sections to stretch.
3. The section is transferred to a dish containing an 0.25% aqueous solution of potassium permanganate for 1 hour (stir solution often). Removal of pigment may be accelerated if the solution is kept at 37 °C.

*Emanuele, P. V.: Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C. 20305.

6. Transfer section to albuminized slide and allow to dry on slide warmer overnight or place in a 60 °C oven for 30 minutes.

7. Deparaffinize and stain as desired.

FORMALIN AND MALÁRIA PIGMENTS

In performing these procedures it must be understood that *no specific time* can be set for the various methods given since much of the results may depend on the length of storage as well as the treatment of the specimen prior to processing. But it must be understood also, that the timing suggested has been based on our experiente and is given as the minimum removal time only.

Both formalin and malarial pigments can be removed quite readily (5 -10 minutes with procedures given) if the tissue specimens are washed for 16 hours in running water prior to exposure to the dehydrating alcohols. If washing is omitted, removal of pigments may require as much as 24 hours.

The following technics serve to remove both formalin and malarial pigments. No satisfactory procedure has been developed to specifically differentiate these pigments in tissue sections by their resistance or solubility in various chemicals. However, several of the methods presented below will remove formalin pigments more rapidly than malarial pigments. Remarks made concerning malaria parasites after the various procedures relate to the staining effect produced with the H&E following exposure of the slides to the different procedures.

METHOD 1

1. Deparaffinize and hydrate to water.

2. Rinse well in distilled water.

BLEACHING SOLUTION

Acetone ..... 50.0 ml
Hydrogen peroxide, 3 % ..... 50.0 ml
Ammonium hydroxide, 28% ..... 1.0 ml

4. Wash well in running tap water and distilled water then stain as desired.

REMARKS. All staining results are similar to method ##I with the mataria parasites *considerably better demonstrated.*

METHOD IV*

1. Deparaffinize and hydrate to water.
2. Rinse well in distilled water.
3. Place in the following solution for 1 hour.

Alcohol, 95% ..... 50.0 ml
Ammonium hydroxide, 28% ..... 15.0 ml

4. Wash well in running tap water and stain as desired.

REMARKS. *This method is* the method of choice in our laboratories because it *removes both malarial* and formalin pigments in 1 hour. The staining qualities are excellent.

METHODS FOR RESTORATION OF BASOPHILIC PROPERTIES

Hematoxylin and eosin stained sections which exhibit poorly stained nuclei even though all stains and chemicals are known to be in propor working condition, could be duo to one of the following reasons:

1. Long storage in acid formalin or other fixative.
2. Over exposure to decalcifying solutions.
3. Dried or burned tissue.

Methods which have been used successfully, in our laboratories, for restoring some of this loss of basophilic property are presented herewith. Because no one method solves any specific problem every time, the list presented is in order of our experiente. However, if, M. any given situation, poor results are encountered in following one method, try another until success is accomplished (Fig. 16 A, B).

METHOD 1

I. Deparaffinize and hydrate to water.

2. Place in a 5/^{1}ı, aqueous sodium bicarbonato solution overnight. (In most instances 4 hours is sufficient).

3. Wash in tap water for 5 minutes.

4. Stain as desired.

METHOD 11

1. Deparaffinize and hydrate to water.
2. Place in a 5% aqueous solution of ammonium sulphide overnight.
3. Wash in tap water for 5 minutes.
4. Stain as desired.

*Washington, Lillian K.: Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C. 20305.

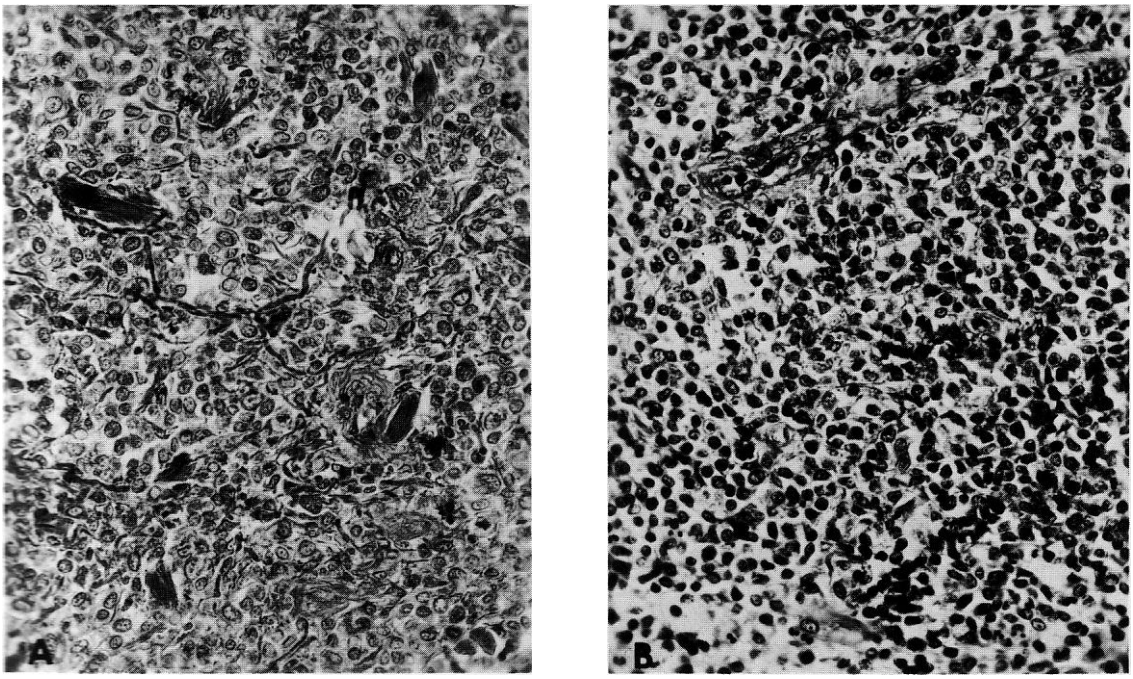


Fig. 16. Comparison of a section stained after overfixation with Zenker's solution (A) and (B) of a section after treatment with basophilic restoration properties Method # I. (A=AFIP 67-3718; B= AFI P 67 - 3719)

METHOD 111

1. Deparaffinize and hydrate to water.
2. Place in 5% aqueous periodic acid overnight.
3. Rinse in distilled water, three changes.
4. Stain as desired.

REMOVAL OF COVERSLIPS

When the need arises for restaining slides it becomes necessary to remove the coverslips and this can be dono in one of several ways. When employing any one of these methods, remember that the older the slide, the longer it will take to remove the coverslip.

METHOD 1 (XYLENE)

1. Place slide in xylene until coverslip detaches of its own accord. (leave slide in xyIene until all of the mounting media is dissolved).
2. Hydrate to water.
S. Stain as desired.

METHOD 11 (HEAT)

1. Heat underside of slide around coverslip edges, by passing over a flame (Bunsen bumer, cigarette lighter, etc.) about 2 times a second. *Do not hold slide directly on*

Place bones in fresh 10% formalin for 24 hours. Make this by -mixing 40 ml of formalin with 360 ml of degassed water. Tip the bone with each change of solution to remove the trapped air froco mastoid and other air spaces.

Place in fresh 10% formalin for 24 hours.

Place in fresh 10% formalin for 24 hours. Bones may be stored in this.

Trim the bone specimen with a circular saw or a band saw. The specimen should be trimmed with vertical cuts made anterior and posterior to the inner and middle ear structures. These cuts are perpendicular to the tentorial ridge. The anterior cut should be as far anterior to the internai auditory meatus as is permitted by the size of block.

To remove bone sawdust, carefully grasp the bone with stout toothed forceps and shake rapidly with a back-and-forth motion in 10% formalin in a tall jar. The motion of a vigorous tremor is best. Turn the specimen 90 degrees and repeat. Turn once again and repeat. Each shaking should be vigorous and should last 15 seconds. Use of a tall jar minimizes splashing.

8. Remove specimen and examine it. Gross pathology notes may be dictated at this time.

Suspend each bone by a string which passes through the tentorial margin, through soft tissue near the glenoid fossa, or around the bone. Either nylon suture material or white carpet thread (preferably linen) that has been soaked in melted paraffin can be used. Suspend the bones so that the toes are at least one inch below the fluid surface when 400 ml of fluid are in the jar. The bottom of the bone should be at least one inch above the bottom of the jar.

Decalcify the bones at room temperatura **in** 1% nitric acid in 10% formalin. Use a separate glass jar for each pair of bones. For decalcification, each jar should contain 356 ml of degassed water, 40 ml of formalin, and 4 ml of nitric acid. Add formalin to the water; then add the acid. Mix carefully to avoid forming bubbles. Transfer each pair of bones to a fresh decalcifying solution daily. When transferring bones to fresh solution, have the fresh solution in a thoroughly washed clean jar standing near the jar containing the bones. Transfer rapidly, to minimize air-trapping. Tip the bones with glass rods to release any trapped air.

Test the decalcifying solution periodically after two or three weeks have passed to determine when decalcification is sufficient. The test is performed just before the bones are transferred to fresh decalcifying solution. Test the fluid that is directly beneath the suspended bone specimen; this has the greatest concentration of dissolved calcium salts when the fluid has not been agitated.

The test is performed as follows: while keeping the upper and of a pipette or glass tube closed with a finger, introduce the pipette finto the jar so that the tip is placed on the bottom of the jar beneath the bones. Allow the fluid to flow up the pipette or tube. The amount needed for the test is 5 to 10 ml. Add this to a fresh solution of testing fluid, made by adding about 5 ml of 5% ammonium hydroxide solution to about 5 ml of 5% aqueous ammonium oxalate solution in a test tube. Exact measurements are not essential. If no precipitate forros, allow test tube to stand overnight before reading the test as clear. For each pair of bones, a record should be kept of the tests, using terms such as "distinct," "faint," "very faint," etc., to describe the precipitate

This is an empirical test to determine an and-poínt for histological sectioning; a more delicate chemical test will still indicate the presente of calcium at a stage when the bone is atile to be sectioned. Human temporal bones usually require 20 to 30 days for decalcification.

11. When decalcification is complete, specimens may be held in 10% formalin until others are ready for the next step.

12. Place in 5% sodium sulfate for 24 hours. For this, dissolve 20 grams of sodium sulfate in 380 ml of water for each pair of bones. Degas just bafore using.

13. Change to fresh 5% sodium sulfate for 24 hours.

14. Wash in either (A) running degassed water, 48 hours, or (B) hourly changes of degassed water during working hours of three consecutiva days.

15. Place in 35% ethanol for 24 hours. Make this and the following dilutions of alcohol with degassed water.

16. Place in 50% ethanol for 24 hours.

17. Place in 80% ethanol for 24 hours.

18. Place in 90% ethanol for 24 hours.

19. Place in 95% ethanol for 24 hours.

20. Place in 95% ethanol for 24 hours.

21. Place in 100% ethanol for 24 hours.

22. Place in 100% ethanol for 24 hours.

23. Place in a mixture of equal parts of 100% ethanol and ethyl ether for 24 hours.

24. Place in a mixture of equal parts of 100% ethanol and ethyl ether for 24 hours.

25. Transfer specimens to nitrocellulose or parlodion solution for beginning infiltration. Nitrocellulose solutions used in these steps should be

28. Transfer to 20% nitrocellulose (or 12% parlodion) for three weeks. Tip to release trapped air bubbles.

29. Transfer to 30% nitrocellulose (or 16% parlodion) for three weeks. Tip to release trapped air bubbles.

30. Imbed in 35% nitrocellulose (or 16% parlodion), in 100 mm diameter Stender dishes of 50 mm depth, with lid sealed with vaseline and held with weights.

Stender dishes for this purpose should have individually ground covers so that the cover fits exactly the individual dish. After receiving the dishes so purchased, mark the top of the cover and the side of the dish with an identifying number so that the two are identified as belonging to a pair.

To imbed, pour 35% nitrocellulose finto the Stender dish and pour a few drops of ether-alcohol o[p]to the surface; put vaseline-rimmed cover on, add weights, and wait for the bubbles to come out (4 - 5 hours). For weights, use two full glass micro-slide boxes (50 x 75 mm microslide size). Then remove the weights and the top, cut the string from the bone, and transfer the bone to the 35% nitrocellulose. Tip to release trapped air bubbles. Add a few drops of ether-alcohol to the surface to remove surface bubbles. Reseal the lid in the vaseline; replace weights on the top.

Four bones (two pairs) can be imbedded in one dish. Note the position of the bones in the dish on a sketch; use the number on the side of the dish as a guide to the positions of the specimens.

31. After the bones have been in the sealed dish for three weeks, remove the weights from the top during the daytime and replace them at night. Do this each day for three weeks, providing that the relativo humidity is 50% or less. If it is greater than this, do not remove the weights. The nitrocellulose should have an even consistency throughout. If a surface crust forros, reseal and replace weights; solvent will ascend in the sealed dish and will soften the crust.

32. When the surface of the nitrocellulose is no longer "tacky" but yields with gentle finger pressure, flood the surface with chloroform. Replace the top, and add the weights. Allow two to three days for the block to harden.

If more than one specimen is in the dish, before adding the nitrocellulose make a mark on the surface of the nitrocellulose near the mark on the side of the glass dish. This will mark the position of this reference point if the block becomes loosened during hardening. This reference point is important in identifying the specimens.

33. Pour off the chloroform. Pour on 80% alcohol, to fill the dish. Replace the cover and let it stand for one day.

34. Cut block out of dish. Cut blocks by scoring V-shaped grooves on the top of the hardened nitrocellulose between the bones in the dish. Continue deepening these grooves, using a thin bladed scalpel, to separate individual blocks.

35. Nitrocellulose blocks should be kept moist *at a11 times* with 80% alcohol.

36. Mount nitrocellulose block on fiber or wood block. The top of the

Remove the fiber or wooden block froco the alcohol-ether container. Pour thick nitrocellulose into the paper container on the top of the block.

Push the nitrocellulose block into the thick nitrocellulose on top of the mounting block quickly, before the mounting medium begins to harden, and push it firmly down onto the grooved surface. Hold it firmly for one minute. Allow it to sit for 5 -10 minutes. Then immerse it in 80% alcohol to harden, handling it by the fiber or wood block, not the specimen. On the following day, the paper can be removed. Store the mounted blocks in 80% alcohol.

37. Cut sections on a sliding microtome at 24 micra, keeping the block wet with 80% alcohol. Alcohol should drip onto the block; the sections on the knife should also be kept moist with alcohol. Select every tenth section for staining. Placa the other nine, in sets of nine, in order on Bantam manifold paper 1#9 strips which are numbered with the section number. The paper strips are wrapped in unglazed paper and then in gauze, tied with a string, and stored in 80% alcohol.

38. Stain the sections overnight in Ehrlich's hematoxylin. Arrange the sections in order in the stain in a large glass pie plate.

EHRLICHS STOCK SOLUTION

Crystals (not powder of hematoxylin)	5.0 gm
Absoluta alcohol	250.0 ml
Distilled water	250.0 ml
Glycerin	250.0 ml
Glacial acetic acid	25.0 ml
Potassium alum, to excess	50.0 gm

To prepare, dissolve the hematoxylin crystals in the absoluta alcohol, add the glacial acetic acid, then add the glycerin. Dissolve the alum in the water, and add the rest. Let stand in a loosely covered, filter-paper capped flask. The paper cap should be lifted off each day and the flask swirled to mix in the precipitated alum. The flask should be large enough to be only partially full. Allow to stand at room temperatura on an open shelf, not in direct sunlight, until ripened (approximately three months). M-hen ripened, the mixture will give off a fruity smell, like Burgundy.

ERHLICH'S HEMATOXYLIN STAIN

Add 10 to 20 drops of Ehrlich's stock to 300 ml of water. The number of drops used will depend on the degree of ripening of the stock. Seldom are more than 20 drops needed. Use this fresh diluted solution for the stain. Because of differences in the stain, it is best to stain a few sections for a trial before staining an entire series.

Stain **overnight.**

EOSIN STOCK

Dissolve 2 gm Eosin Y in 50 ml of 95% alcohol and 150 ml of water.

EOSIN STAIN

Diluta 6 ml of Eosin Y stock with 150 ml of water. Stain for Eive minutes.

42. Place in 95% alcohol for 3 minutes.

43. Place in 95% alcohol for 3 minutes.

44. Place in steam-distilled pine oil for at least 15 minutes.

45. Take sections from pine oil and place on slide. Blot the section dry with lintfree Whatman #I chromatography grade filter paper. Press heavily to remove the oil.

46. Place approximately 2 drops of Damar balsam on a coverglass and place coverglass on the slide. Press gently to remove bubbles. With #1 coverglass, the margins of the coverglasses will bend down and adhere closely to the microslide, decreasing the chance of air coming under the coverglass and forming bubbles. Use this thickness, rather than #2 coverglasses.

47. Label slides with diamond marker.

SUPPLIES. Microtome. **American Optical, Sliding Microtome #860, with 250 mm Thomas-Schmid knife, É#950, and Ajustable Knife Clamp AO cat. ##862.**

Knife Sharpener. **Microtome Knife Sharpener, Thomas-Fanz, for 250 mm knife, A. H. Thomas, cat. #É7203.**

Microslides. Red label corrosion-resistant glass microslides, 50 x 75 mm Arthur H. Thomas, cat. ##7033.

coverglasses. **Red label coverglass #É1, 35 *x* 62 mm, A.H. Thomas, cat. #7024. *Do not* use #2 coverglasses.**

Stender Dishes. 100 mm diameter x 50 mm deep and 130 mm diameter x 50 mm deep, A. H. Thomas, cat. #4514. Stender dishes should have individually ground covers so that cover matches the individual dish. After receiving dishes so purchased mark the cover and the sido of the dish with an identifying number so that the two can be identified as belonging to the same pair.

Whatman #1 Chromatography Paper. **A. H. Thomas Company, Vine & Third**

A PARAFFIN TECHNIQUE FOR PROCESSING EYES

This is a technique for processing eyes in a wax medium which permits production of completed sections of whole globos ready for reading by the pathologist one «veek after enucleation. Adaptation is made of various accepted techniques used in fixing, processing, cutting, and staining routine tissue.

The use of this technique also makes possible a greater number of special staining procedures specifically adapted to eye tissue. This in turn aids in the more difficult diagnosis or any further research that may be indicated.

FIXATION. The enucleated eye should be, fixed immediately in 10% formalin which has been buffered with sodium phosphate monobasic and sodium, phosphate dibasic (anhydrous), (see page 3) in a volume 20 to 25 times that of the eye. Fixation is usually complete in 48 to 72 hours and the specimen is ready for processing or shipment as the case may be. *cutting windows or injecting the fixative finto the globe is neither necessary nos desirable.* The technique after receipt of the globe in the labora-ton- follows:

1. Wash in running water for 8 -24 hours to remove fixative.
2. Place in 60/[1]'c, alcohol until ready to cut, this restores much of the normal color m--hich has been dulled by the fixative; it becomes the first step in dehydration; and it is a good storage solution.
3. Write a good complete description of the specimen including the externai appearance, measurements, general aspects of the globe, and note any scars of injury and/or operation.
4. Open the globe with a flat razor blade,[2] starting several millimeters froco either sido of the optic nerve and passing through the cornea just outside the Timbus. (The conter block containing the pupil and optic nerve is that portion used for processing while the two sidos, calottes, or caps, are held in alcohol for further possible use). Deçcribe internai structures of the conter portion indicating known or suspected lesions

svell as abnormal appearance. Also incorporate any special instructions to enable :-áe technician to demonstrate any abnormal lesions or conditions in relation to the normal structures.

5. Orientation and embedding instructions should be written on an identification -a,_a which accompanies the specimen through the processing. Place specimen to be processed in 80% alcohol, second step of dehydration, to await processing.
6. If calcified material is observed on "gross" sectioning, the specimen should 3-- placed in decalcifying fluid for several days. The fluid of choice for eye tissue is sodium citrato and formic acid solution (see page 8). Following the use of the decalLí-N-ing solution the specimen must be washed in running water for 24 hours before i.,her processing.

7 Processing by an automatic processos or by hand, depending upon either

	Overnight (automatic)	By hand (for *largo pieces) eces)*
-Alc,ohol, 95/11c - **Alcohol, 95%**	3 hours 2 changes, 1 hour each	8 - 24 hours (several changes)
hol. 100%	3 changes, 1 hour each	8 -24 hours

Chloroform	1 hour	*5* hours
Chloroform	*2* hours	(several changes)
Paraffin (Bioloid, wax of choice for eyes)	*2* changes, 1 hour each	*5* hours (several changes)
Paraffin	*2* hours	
Paraffin (under vacuum)	*30 - 45* minutes	*30 - 45* minutes

8. The embedding is comparable to general embedding prodedures using the multiple embedding technique (see page 13) following specific instructions on the individual tags.

9. Blocking, boxing and holding for completion will be as the accepted plan in the individual laboratory.

SECTIONING. *To obtain satisfactory sections the following instructions niust be followed closely:*

a. Cut at *8* microns.

b. Placo block with scleral sidos parallel to knife, cornea and nerve on either sido

c. Orient block to get as near a complete section as possible without excessiva rough cutting so that the required sections will be through the nerve and pupil as well as demonstrating known or suspected abnormal features.

d. Expose the tissue and soak for several minutes, using a piece of cotton saturated with warm tap water. This aids in cutting a cross section of the globo with minimal tearing through lens, gelatinous exudate, and/or blood.

e. Chill both the block and the knife with ice and cut using a smooth slow turn of the microtome wheel.

f. Use two flotation baths at this point: one of distilled water at room temperatura, upon which the ribbon of sections is placed; and a second maintained at *55'* to *55.5 °C* approximately 10 degrees higher than normally used for general tissue flotation, to which has been added gelatin adhesive, *3* teaspoonful of *5%* gelatin solution per 1000 ml of water. Upon this flotation bath each individual section is stretched to conform in size and chape with the tissue in the original block.

g. At the Glose of each day's work, carefully clean with detergent and dry the flotation bath that contained the gelatin, to insur[g]i against bacterial growth which could adhere to slides and thus cause an artif act giving a confused diagnoses.

STAINING. After drying, the completed sections are ready for staining as desired, routine H&E and by special staining techniques.

REFERENCES. 1. Ballou, E. F.: *Amer. J. Med. Techn. 32:287 -291, 1966.*

2. Ackerman, L.V.: *Surgical Pathology,* 3rd ed., St. Louis, The C.V. Mosby Company, *1964, pp. 1218 -1219.*

TRYPSIN DIGESTION TO DEMONSTRATE RETINAL VESSELS

FIXATION. 10% buffered neutral formalin. Remaining portions of the globos (see page 53) which have been in alcohol should be placed in

SOLUTIONS

0.15 M TRIS BUFFER (pH 7.8)

Tris Buffer, (Sigma* 7 -9, M. W. 121. 136) ..... 18.17 gm
Distilled water ..... 1000.0 ml
Hydrochloric acid, concentrated, (to adjust pH if necessary)

TRYPSIN DIGESTION SOLUTION

Trypsin, (Difco t 1:250) 3_0 gm
Tris Buffer ..... 100.0 ml
Merthiolate (as a preservativo) ..... 1 pinch
Make fresh for immediate use.

PROCEDURE. Material usually used is taken from the two sidos of the globo, or he calottes, after the conter portion containing pupil and optic nervo has been processed for routine pathology evaluation.

DISSECTION OF THE RETINA. Work under the dissecting microscope with the calotte immersed in distilled water in a finger bocal. Starting at the posterior polo, Nvith the "heel" of the dissecting needle, separate the retina propor from the choroid'Z.Clera which is held firm with jeweler's forceps. Separate at the pars plana. When the choroid adheres to the retina, it is often easier to partially digest both before attemptme to separate them. (An initial digestion of the entire calotte sector for 10 - 20 minutes may be helpful).

DIGESTION. Suspend the retinas in the trypsin digestion solution at 37 °C for 1 2 to 1-1/2 hours. Incubation time varies with each eye. Terminate digestion when _mie médium becomes cloudy and the tissue shows signs of disintegration, by trans-Mi10[1] to distilled water. This is accomplished by picking up the retina on the heel ,À the dissecting needle. The vessels are surprisingly strong.

PREPARATION OF THE RETINAL VESSELS. Work with the dissecting microscope, and vith the retina submerged in a finger bocal of distilled water. The internal limitmlz membrane can be peeled off in one sheet, by careful manipulation. Hold the internal -miting membrane with a jeweler's forcep, and disengage the retinal vessels from

with the heel of the dissecting needle. (The point of the needle may adhere to and --c--ar the vessels). Grasp the interna] limiting membrane at a point close to the nine -À separation. The network of vessels can be freed of retinal tissue by gentio shaking. lf undigested tissue remains, it may be necessary to incubate longer. The final retinal vessel preparation will appear as an open meshwork. The veins and arteries branch and fan out, and are interconnected by a capillary plexus.

MOUNTING. Replace the water with clean distilled water. Dust should be skimmed om the surface, float the vessels on a clean slide, and with the slide preparation partly

-- iersed, use the heel of the dissecting needle and the surface tension of the water ar-range the vessels in their original configuration. Air dry.

STAINING. PAS-hematoxylin for demonstration of vessel walls and their cellu-luclei. (Arteries stain more intensely than veins).

Hematoxylin-eosin for visulization of red blood cells. (It is necessary to increase - Staining time in eosin).

REFERENCE. Kuwabara, T., and Cogan, D. G., *Arch. Ophthal.* 64:904-911, 1960

STAINING METHOD FOR RETINAL VESSELS (PAS)

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. See page 53.

SOLUTIONS

M/5 SODIUM ACETATE

Sodium acetate $(NaC_2H_3O_2 \cdot 3H_2O)$ ..... 13.6 gm
Distilled water ..... 500.0 ml

PERIODIC ACID SOLUTION

Periodic acid ($HIO_4 \cdot 2H_2O$) ..... 0.8 gm
Distilled water ..... 20.0 ml
Dissolve and add:
M/5 sodium acetate ..... 10.0 ml
Alcohol, 95% ..... 70.0 ml
Prepare fresh each time.

REDUCING RINSE SOLUTION

Potassium iodide ..... 2.0 gm
Sodium thiosulfate ..... 2.0 gm
Distilled water ..... 40.0 ml
Dissolve and add:
Alcohol, 95% ..... 60.0 ml
2N hydrochloric acid ..... 1.0 ml
Prepare fresh each time.

SCHIFF'S LEUCOFUCHSIN SOLUTION

(see page 159)

COLEMANS FEULGEN SOLUTION

(see page 159)

MAYER'S HEMATOXYLIN

(see page 33)

STAINING PROCEDURE

1. 70% alcohol for 5 minutes.
2. Oxidize in periodic acid solution for 5 minutes.
3. 70% alcohol, several changes, 2 minutes each
4. Reducing rinse solution for 5 minutes.
5. 70% alcohol, several changes, 2 minutes each.
6. Rinse in distilled water.
7. Coleman's or Schiff's leucofuchsin solution for 35 - 60 minutes.
8. Running tap water to develop color for 10 minutes.
9. Mayer's hematoxylin for 10 minutes.
10. Wash in running water for 15 minutes.

11. Dehydrate in. 95% alcohol, absoluta alcohol, and clear in xylene, 2 changes each.

12. Mount with Permount or Histoclad.

RESULTS

Vessel walls - magenta

Nuclei - blue

REFERENCE Hotchkiss, R. D.: *Arch. Biochem.* 16: 131-141, 1948 (AFIP modification). Copyright by Academic Press.

SECTIONING UNDECALCIFIED TISSUES

There may be times when it becomes particularly essential that sections be made of undecalcified tissues so that no portions of the bone architecture are changed or obliterated by any decalcifying procedure. Under such circumstances either of the two following methods may be employed:

SECTIONING OF UNDECALCIFIED TISSUES USING GILLINGS-HAMCO* THIN SECTIONING MACHINE

FIXATION. Any well fixed tissue, however, 10% formalin preferred. After fixation, cut specimen to desired thickness (5mm in thickness is suggested).

DEHYDRATION.

Place specimen in the following solutions for times indicated.

Alcohol, 80% ..... 2 qw hours

Alcohol, 95% ..... 2 hours

Alcohol, 100% ..... 2 hours

Acetona ..... 1 hour

Remove specimen froco acetone and allow the acetone to evaporate, 15 minutes.

IMPREGNATION. Place specimen in a jar of castolitet (plastic without the catalyst) with enough plastic to cover specimen, impregnate under vacuum at least 24 hours. *N·Ote.* This plastic can be re-used, after specimen is removed, if plastic is placed in -he refrigerator.

CASTOLITE PLASTIC SOLUTION

Castolite .....100.0 ml

Catalyst..... 5 drops

Mix just bafore use. Stir carefully until well mixed, try to avoid bubbles.

BLOCKING. Pour about 1/4 inch castolite plastic solution into a mold made with Re·-nolds wrap or other suitable material. Place tissue in remaining catalyzed plastic ready to be poured into mold. Allow mold to stand at room temperature for 2 hours then place in a 60°C oven until polymerized but with top still tacky. This providas the

~CílIffigs-Ha~ sectioning machine, Hamco Machines, Inc., Rochester, N.Y. 14607

C 1 Washington, D.C. 20012 --stol[i]te (liquid casting plastic), Read Plastic, Inc., 317 Cedar Street,

at room temperature after the block has been removed from the mold. (Refrigeration will aid in removing block from the mold). Trim the hardened block with a saw, unless the mold was made to exactly fit the block.

Using Duco cement, attach the plastic block in the desired position to a plastic or masonite base. Thirty minutes in a 60 °C oven, followed by room temperature cooling is all that is required for a firm attachment. The base and attached specimen is, screwed to the machine specimen mount.

SECTIONING. Regulate water supply making certain it is steady bafore the cutting is started. While sectioning keep constant check to be sura the water pressure does not vary. Too little water wi11 burra the tissue. Align block and set for section thickness desired. Start machine.

When sectioning is completed, turn off the machine and water and disengage the blade. Extreme cara is required in handling the section to prevent fracturing. Blot section between filter papar, and store in dust free container until ready for mounting.

MOUNTING. **Dehydrate in 95%, absolute alcohol and clear in xylene, 2 changes each. Mount with Permount or Histoclad.**

RESULTS. The structure of teeth and cortical bone is clearly demonstrated.

REFERENCE. Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, D.C. 20305.

PREPARATION OF HAND GROUND SECTIONS OF CORTICAL BONÉ

FIXATION. Any well fixed tissue, however 10% formalin preferred.

TECHNIQUE.

1. **Cut bone finto thin slices with hand or band saw.**

2. **Place bone in a thick soap solution (strong laundry soap) and boil for 4 hours to remove organic material.**

3. **Wash in running water overnight.**

4. **Place the bone in absolute alcohol for 7 hours.**

5. **Suspend bone over ether, benzene, or chloroform for 48 hours to remove fats. Change solution twice during this step.**

PARAFFIN METHOD FOR LARGE BONÉS AND WHOLE ORGANS

The following method is used for sections of bone or organs which measure as as 5 inches in length and 1/2 inch in thickness.

FIXATION. Bone and large soft tissue specimens should be fixed in 10% buffered in a volume of 20 to 25 times that of the specimen, for 5 days.

DECALCIFICATION. After they are thoroughly fixed, the blocks are tied in gauze a label with the aceession number, paraffin dipped for protection, is attached. bag is suspended in a large quantity of decalcifying solution. Stirring or agitation of the fluid hastens the decalcifying process.

Decalcification may take from 3 -14 days depending on the size of the specimen. Change decalcifying solution frequently (at least every other day) until decalcification is complete. When decalcification is complete wash the bone in running water 24 hours. Tissues are then transferred to 80% alcohol, bafore routine hand processing is employed.

PROCESSING. A11 large specimens must be processed by hand and the following schedule is suggested:

PARAFFIN METHOD

1.	Alcohol, 80%, 2 changes	12 hours each
2.	Alcohol, 95%, 2 changes	12 hours each
3.	Alcohol, 100%, 2 changes	8 hours each
4- Chloroform, 3 changes	4- 5 hours each	
-S- Paraffin (Paraplast) 4 changes	8 -10 hours each	

6. Embed. For proper embedding, flatten tissue with gentle downward pres**while** rubbing an ice cube across the bottom of the embedding pari (see pape 13).

Attach paraffin block to a fiber or wooden block by melting the back of the with a hot spatula and pressing the block and holder firmly together. Be certain **:he** back of the paraffin block is even to prevent any air spaces between the parij- and the mounting block which will produce vibrations during the cutting stage.

---erse the fiber block containing the paraffin block, in a pari of tap water. Do not o-o rapidly or the tissue block will crack away from the fiber block.

S. The blocks are sectioned on the Sartorius modal 39 or any microtome which aeconimodate large tissue blocks.

The largar the block being cut, the more tendency for the sections to roll-up **thev** come from the knife edge. In this case, they are removed in the rolled state a room temperatura water bath. Transfer the rolled section on a glass slide, with c- and of the roll uppermost, to the conventional 40-45'C flotation bath. Aided th-e heat of the water, unroll the section with a gentle pushing pressure using a camel's brush, until the flattened section will float free on the water's surface. Pick up the **--.:in** on a labeled slide which has been treated with Mayer's albumin section adhesive.

10. These slides are thoroughly dried in a 37-40 °C drying oven and are then for staining.

STAINING PROCEDURE. For ,the staining of sections on slides measuring as much 5 x 7 inches, animal jars 8 inches tall and 8 1/4 inches in diameter are used. The

steel rack. For routine staining any one of the methods presented on pagos 36 -40 can be used satisfactorily. An increase of time in most solutions may be desirable, but only by experiente and observation of a given slide during the staining procedure, can dictate this necessity.

Any of the many special stains available can be performed on sections of this size by flooding the slide with the various special staining solutions and allowing them to stand for the desired time. When using this technique it is well to placo the slides on rods across a pan or staining dish to prevent spillage of excess solution.

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D. C. 20305.

METHODS FOR PROCESSING ORAL TISSUES

TEETH

Propor fixation of a tooth requires the clipping of the apical third of the root or roots immediately after extraction from the host. This simple step permits the fixation solution to penetrate the tooth completely.

FIXATION

1. 10% buffered neutral formalin for 72 hours minimum; wash in water for 4 hours.
2. Place in one of the following solutions until decalcification is complete, changing the decalcifying solution daily for best results. (10 -12 days in formic acid solution. Approximately 5 days in nitric acid solution).

FORMIC ACID SOLUTION

Formic acid, 90% 5 0 m1
Distilled water .....95.0 ml

NITRIC ACID SOLUTION

Nitric acid, concentrated .....10.0 ml
Distilled water .....90.0 ml

Decalcify in refrigerator. (20 °C if this method is used).

3. When decalcification appears to be complete, cut a thin slice froco each sido of the tooth and return to decalcifying solution for an additional 24 hours to insure complete medial decalcification, the end point of which is determined by any of the methods listed on page 10.
4. Wash in running water overnight (16 hours).
5. Place in 60% alcohol for 24 hours, followed by 80% alcohol for 48 hours.
6. Proceed with routine automatic tissue processing schedule (soe page 16).
7. Embed and cut as desired.

SPECIAL CUTTING INSTRUCTIONS

It is obvious that teeth will present problems not found **in** other tissues duo to their extremely hard composition, even after complete decalcification.

intervala during the rough cutting process prevents loas of or damage to tissue (see page 26).

Sections of teeth have tendency to spread to their original size when placed on the water bath, but are bound by the surrounding paraffin. In this case "slit" the paraffin on all four sides with a suitable instrument to allow for natural expansion to original chape. On a section of a particularly stubborn tooth the distention may be aided manually by pulling gently at opposite ends of the tooth until it is completely flat and smooth. Float se!ctions onto albuminized slides and stain as desired.

JAW BONÉS

IXATION

1. 10% buffered neutral formalin preferred. Place specimens in a volume 20- [2]5 times that of the specinen for 10-14 days. Change solutions *3* times during this Deriod. *Note*. In animal research it is best to perfuse the animal after which the jaw cone is placed in formalin solution for 1 week.

2. Wash in running water overnight.

3. Place in decalcifying solution until decalcification is complete. (Human size _:M-W bones decalcify in 6 weeks to 2 months when formic acid is used; 4 - 6 weeks for nitric acid). Change solutions daily for the first *3* weeks followed by changes every ckher day for the remaining period. Test with the chemical decalcification end point procedure given on, page 10. If not completely decalcified place in 5% aqueous disodiumethylenediaminetetraacetate for *3* days followed by washing in running water for 2 hours. Re-treat with decalcifying solution for 2 days. When decalcification is compk--te. cut a thin slice froco each side of the specimen to assure complete penetration.

PROCESSING

Alcohol, 60%	2 days
Alcohol, 80%, *3* changes	.5 days
Alcohol, 95%, *3* changes	7 days
Alcohol, *100%, 3* changes	*3* days
Alcohol, 100%, 3 changes	3 days
Chloroform, 2 changes	1 hour each
Chloroform, 2 changes	16 24 hours each
Paraffin of choice, 3 changes	3 hours each
Paraffin of choice, under vacuum	2 hours
Paraffin of choice	*3* hours
Paraffin of choice	*3* hours
Paraffin of choice under vacuum	2 hours

EMBED. See page *13.*

CUT. See page 59.

STAIN. As desired.

SOFT TISSUES

RXATION

1. Tissues should be cut in half before being placed in a fixative of choice (10% ---ered neutral formalin for 30 minutes). Then grosa to desired size, and place in

fresh formalin fixative for complete and propor fixation. *(12-24* hours, depending on size). Small pieces, not to exceed *4* mm in thickness, are fixed adequately in *24* hours. *2.* Wash specimen in tap water bafore processing.

PROCESSING

Alcohol, 60 1% ..... 8 hours
Alcohol, *80%* ..... 24 hours

Process on an automatic tissue processos, or by hand, following the schedule suggested on page 16.

EMBED. See page 13.

CUT. See page *25.*

STAIN. As desired.

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, *D.C. 20305.*

CELLOIDIN METHODS

celloidin or nitrocellulose is, one medium used for processing hard tissues such as bone and teeth as well as for largo sections of whole organs. Use of this medium for processing eye tissue and whole globos had been very successful, however, because of the time required by the method, most laboratories prefer to use the paraffin technique for routine diagnostic work. When the block is hardened to a density which permits cutting, sections can be produced which are free froco compression, distortion, and yet are thin enough to be studied under a microscope.

Nitrocellulose is the forro of cellulose used to make celloidin, and is available in three grades:

Gun cotton - Cheapest, but contains enough explosiva to be potentially dangerous.

Nitrocellulose - Refined, but still containing explosives which requires shipment in water or low grade alcohol.

Parlodion* - Most expansivo. Most explosives removed. Shipped in dry strips.

The disadvantages in the use of celloidin are overbalanced by the advantages as can be observed by the following:

Disadvantages 1. The procedure is time consuming.

2. The celloidin attracts moisture very quickly on exposure.
3. The use of special stains is very limited.

Advantages *1.* Less shrinkage in tissue.

2. Relationship of component parts is preserved with little distortion.
3. Excellent for the study of embryos.
4. Largo, compact bones and whole organs section with no compression or shattering.

*MaIlinckrodt's proprietary terra for the purified pyroxylincelloidin.

The solvent is equal parts of absolute alcohol and ethyl ether. solution is brought about by the following simple steps:

1. Add alcohol 100% to the desired amount of Parlodion and let stand over mght.

2. Add the ether the following

Per cent celloidin desired	*Parts 12% celloidin*	*Equal parts Absolute Alcohol and Ethyl Ether*
10	5	1
8	4	2
6	3	3
4	2	4
2	1	5

FIXATION. 10% buffered neutral formalin. Wash in running water for 16 hours --ore further processing.

DEHYDRATION. This step is accomplished with the use of ascending percentages alcohol, Icohol, starting with 80% and allowing sufficient time for complete diffusion through —e block of tissue in each solution. With hard pieces of tissue it may be advantageous change each percentage of the dehydrating solutions at least twice during the pro-
55
in[g] lince removal of all water is necessary to obtain complete impregnation with __me celloidin. The last dehydrant should be the solvent for the celloidin and the tissue -nould not be allowed to remain in it longer than 24 hours.

Depending upon the type of tissue being processed, each piece should be wrapped a single layer of gauze and fastened with dental floss attached to a label of sufficient ---no-th to allow suspension in the container holding the processing solutions. This permits complete and even penetration throughout all areal of the specimen.

IMPREGNATION. Done by using increasing percentages of the celloidin up to and duding 12% which also becomes the embedding medium. The tollowing tables will z:ve the suggested times of dehydration and impregnation for each of the techniques.

5. Celloidin, 4/$^{01}_{0}$..... 2 to 3 days
6. Celloidin, 8% ..... 2 to 3 days
7. Celloidin, 12% ..... 2 to 3 days
8. Embed. See below.

DRY CELLOIDIN METHOD

1. Same as wet celloidin method through step 7.
2. Place mounted tissue in cedarwood oil and chloroform equal parts .....24 hours
3. Cedarwood oil 3 parts, chloroform 1 part .....24 hours
4. Cedarwood oil until ready to cut .....24 hours or longer

EMBEDDING AND MOUNTING ON FIBER BLOCKS. Place oriented tissue, with the desired cutting surface up as Glose as possible to the bottom of Stender dishes containing thick (12/^{07}0 celloidin to avoid excessiva handling and thus minimizing the formation of air bubbles. The Stender dishes are placed under a sealed bell jar until all of the air bubbles have disappeared, approximately 4 to 6 days. Remove from bell jar and loosen covers of dishes gradually to allow slow hardening of the celloidin by evaporation of the alcohol-ether solvent. Check daily for the proper consistency for cutting into the individual blocks; hard and firm to the touch with slight resiliency upon increased pressure. Cut into individual blocks; tagging with correct numbers. With same surface down, trim into square blocks with parallel sides and affix with thin celloidin to wood or fiber blocks of a size to support all of the tissue block, keeping the amount of celloidin between the tissue and the block at a minimum in order to reduce vibration at the time of cutting. Place in a covered dish in which has been placed a piece of cotton saturated with chloroform, thus using the chloroform vapors for hardening, about 2 hours.

For wet celloidin cutting, remove blocks froco chloroform vapor, placing them upside down in 80% alcohol for at least 24 hours to harden to cutting consistency. Blocks and sections may be stored in 80% alcohol indefinitely.

CUTTING (Wet Celloidin). Place the knife at a 30' angle in relation to the oriented block. *Block and knife must be kept wet with 80%alcohol* by use of a drip bottle, a piece of cotton or a 1" paint brush. Failure to keep the specimen wet will cause shrinkage, and result in thick and thin sections. Irregular sections may also be caused by a loose set screw on the microtome, or bubbles trapped between the embedded tissue and the fiber block. Cover the specimen with cotton wet with 80% alcohol while working with cut sections. Sections to be stained are removed froco the knife with the finger and placed in 95/$^{07}_{6}$ alcohol to straighten. Sections to be stained are floated on two inch squares of smooth filter paper to be wrapped in a package for future work if necessary. For serial work, arrange sections in order on wet knife, pick up by placing a numbered strip of filter paper on the, sections and carefully pull them from the knife. Place face up in a dish of 80% alcohol. This is an easy way to pick up all sections for storage. Cover the top sheet of exposed sections with a slip of filter paper bafore wrapping. Mark with India ink the identifying number on a similar size piece of onion skin paper and cover the number with Duco cement. Wrap sections and block froco the same tissue and tia with dental floss and store in 80% alcohol. The dry celloidin method is used for eyes only, for details of this technique see the original article: Ballou, E. F.: ***Amer.*** *J. Med. Techn.* 14:202-206, 1948.

ROUTINE CELLOIDIN STAINING PROCEDURE

Staining of all types and sizes of tissue can be accomplished with the following procedure. It may be necessary however, for the time to be increased in certain steps, especially in the hematoxylin and eosin solutions. This change in the procedure can only be determined by the size of the specimen being stained and by microscopic obser-- at
ion of the staining qualities.

1. Float sections from 80% alcohol on a glass slide into distilled water.
2. Pick up sections with a glass rod and stain in Harris' hematoxylin (without acetic acid) for 20 minutes.
3. Wash in tap water to remove excess hematoxylin.
4. Differentiate sections individually in a 0.5% acid alcohol solution until celloidin is olear. (HCL 0.5 ml, 70% alcohol 100 ml). Stop the differentiation in tap water bv transfer of sections to water on a slide.
5. Rinse in 95% alcohol until celloidin is clear.
6. Float sections into a weak ammonia water for "bluing."
7. Wash in water and check for nuclear detail with a microscope.
8. Stain in 1 To alcoholic eosin for 30 - 60 seconds.
9. Rinse in 95% alcohol.
10. Rinse in 95% alcohol until clear.
11. Placo in carbolxylene solution or isopropyl alcohol for flattening of the section.
12. Pick section up on numbered slide. Rinse in 2 changes of xylene.
13. Mount with Permount or Histoclad. Small weights are sometimes employed keep the section flat until Permount or Histoclad has hardened.

RESULTS

Nuelei	- blue
Cytoplasm	- pink

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, ,'--asffington, D.C. 20305.

35 MM FILM METHOD

FIXATION. Any fixative may be used.

TECHNIQUE. Paraffin.

SOLUTIONS. Krylon* or Sherwin Williams Plastic Spray Enamelt

'-QUIPMENT. Film: 35 mm Cronar, P 40 B Leader Film*;

Reel: Nikor auto load, from any photographic supply house.

Film holders to hold film in position while viewing film strips on microscope ~e not commercially available. They can be fabricated and consist of a 3 -1/4 x 4 inch _-asj, plate, two 3 x 1 inch slides and four 22 x 40 coverslips and Duco cement (Fig. A strip of paper approximately 1 mm wider than the film is placed in the conter te long axis of the glass slide (the paper can be kept in placo with masking tape). four coverslips are butted against the edge of the paper with Duco cement, the

Inc., Norristown, Pa. 19404
- i flHams, 1325 14th St., N.W., Washington, D.C. 20005
, -wi-'-i ~S
_= L du Pont de Nemours & Co., Photo Productions Dept., Wilmington, Del. 19898

two 3 x 1 slides are placed and cemen[i]ted on top of the coverslips and must extend inwardly 2 mm, over the coverslip edge.

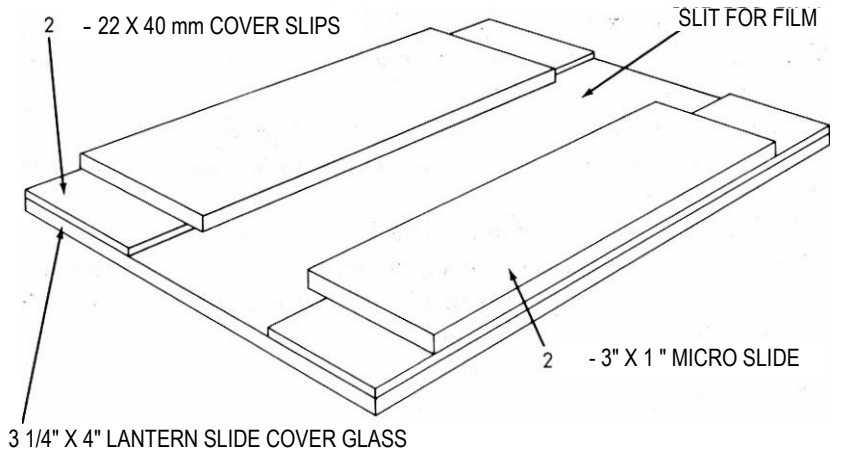


Fig.17. Fabricated film holder for viewing film strips under the microscope.

PROCEDURE

1. Cut paraffin sections in the usual manner and float ribbon onto flotation bath.

2. The film is maneuvered under the ribbon and picked up as on a slide until the entire film is filled. Label film with a diamond pencil or lab ink and placo in a *56* °C oven for 30 minutes to dry.

3. Load film into a 35 mm developing reel (three strips of *18* inch length can be accommodated on one reel). Stain as desired.

4. After staining, one strip of film is removed at a time froco the clearing xylene and placed face up on a blotter. It must not be allowed to dry. *Note:* The film must lie flat because buckling will result in an uneven coat of plastic. Metal weights may be placed at each end of the plastic to keep it flat.

5. The plastic spray can is held approximately *6* inches above the film and the spray is applied quickly until the surface appears clear and smooth.

6. The plastic is allowed to harden for 10 minutes. The plastic spray is repeated twice and left to dry. The strips may be ready for microscopic examination after 2 hours.

7. If necessary, the plastic coat may be removed by placing in xylene for 10 minutes. This step necessary when spray is uneven.

PLASTIC FILM METHODS

The use of plastics, *Cyclon-Lack, farblos Nr. 10830, lufttrocknend*,* Diatext and others, as media for mounting stained microscopic sections in the forro of plastic =ilms.can be utilized easily and advantageously for the preparation of serial sections, t-tudv sets, large sections, removal of multiple sections froco one slide and conversion to several slides, and smears as well as repair of broken slides. Utilization of this method is most practical when considering the problem of storage. Wherein large slide cabinets are used to house one thousand slides, the same number of sections in plastic film, can be maintained in a small card file.

FIXATION. Any fixative.

TECHNIQUE. Paraffin sections and smears.

SOLUTIONS. Plastics as suggested.

::ROCIEDURE

1. Cut sections and place on the flotation bath in the usual manner.
2. Pick up sections on slides and place on the slide warmer overnight or 60 °C oven for 30 - 45 minutes.
3. Deparaffinize and stain as desired.
4. Dehydrate in 95% alcohol, absolute alcohol and clear in xylene.
5. Leave in xylene until ready for plastic.
6. (1) *Either* dip slide in plastic, wipe the underside of slide free of plastic and place on applicator sticks, (Fig. 18), *or*

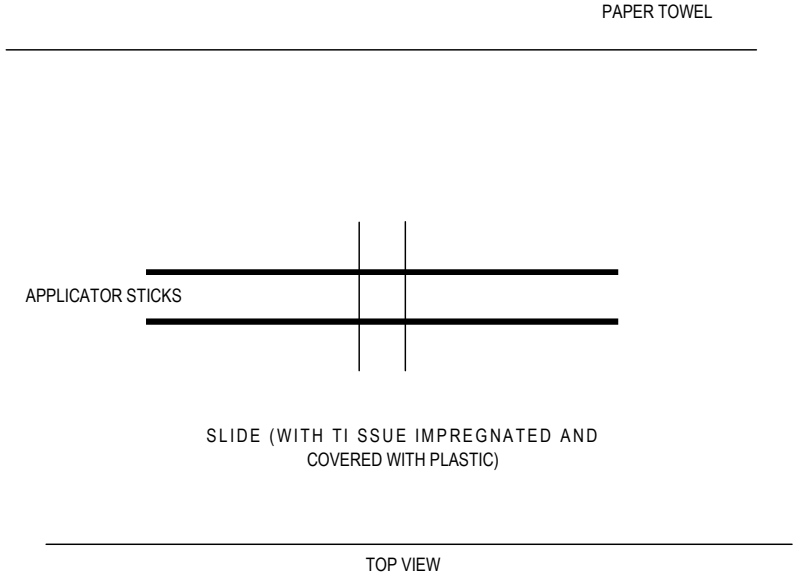


Fig. 18. Plastic drippings

(2) Place slide saturated well with xylene on the applicator sticks, (Fig.18), immediately, before the section dries, pour the plastic on the center of slide allowing -o spread evenly to all sider.

a

--don-Lack, farblos Nr. 10830, lufttrocknend, Lack-u-Farben Fabrick, Bosshard and Co., Zurich, OberhkOn.

60201. - -mex. Scientific Products Inc., 2020 Ridge Ave., Evanston, 111.

7. Dry thoroughly for 2 to 24 hours.

8. Incise film along the edges of the slide with a sharp, instrument.
9. Soak slides in *cold* water for 2 hours.

10. Peel film from slide gently and firmly. (If film does not come off easily, return slides to the cold water for additional soaking).

11. Dry film between filter paper.

12. Number film with India ink and store between sheets of tracing paper, cut in the appropriate sizes.

SECONDARY MOUNTING PROCEDURES

1. Cut sections from the plastic film with scissors or with a razor blade.

2. Place on a small drop of Permount on the slide.

3.* A second drop is placed upon the film and the coverslip applied immediately. *Avoid air bubbles.

MICROSCOPY. For microscopy, the film is placed on a glass slide, slightly larger than the film. Good optical quality and minimal thickness are required for good results.

For visual observation, regular dry systems can be used, or oil immersion objective can be used directly on the film. For optimal results, a drop of oil should be placed underneath the section as well as on the top. Do not use xylene or other solvents to remove oil immersion from film since they will dissolve the plastic. 011 can be wiped off with a clean cloth.

REPAIRING BROKEN SLIDES. Slides sent thru the mail become potential broken slides and many are broken. The use of plastic to repair broken slides lias greatly aided the pathologist since no time or expense is lost in corresponding with the contributos requesting more material. By utilization of the plastic technic, broken slides are repaired as follows:

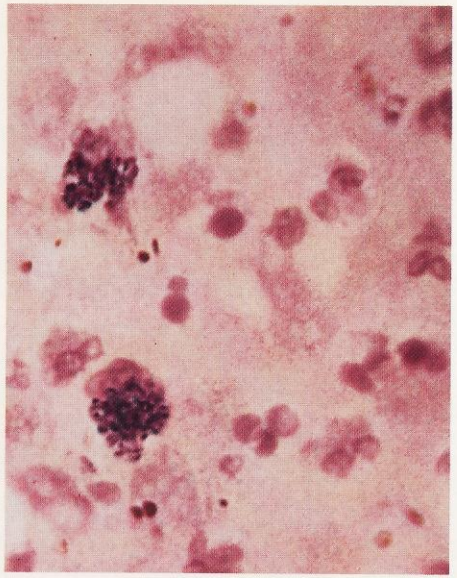
1. Recoves all or most of the pieces from the broken slide, especially those pieces which contam the tissue section.

PLATE 1

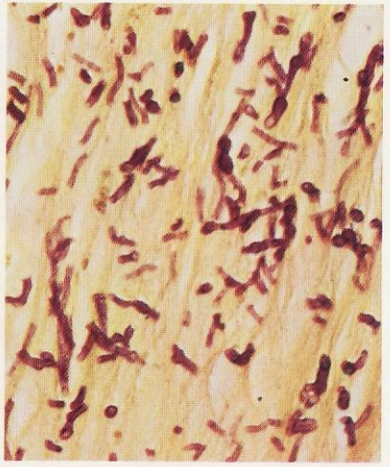
A. Chorioretinal lesion in granulomatous uveitis revealing *Treponema pallidum*- Warthin-Starry Stain: spirochetes—black; background—brownish yellow. (From AFIP Exhibit No. 907. Accession No. 114775.)

Acute endophthalmitis after iridectomy. MacCallum-Goodpasture stain; Gram-positive cocci—blue; other elements—various shades red to purple. (From AFIP Exhibit No. 907. Accession No. 861581.)

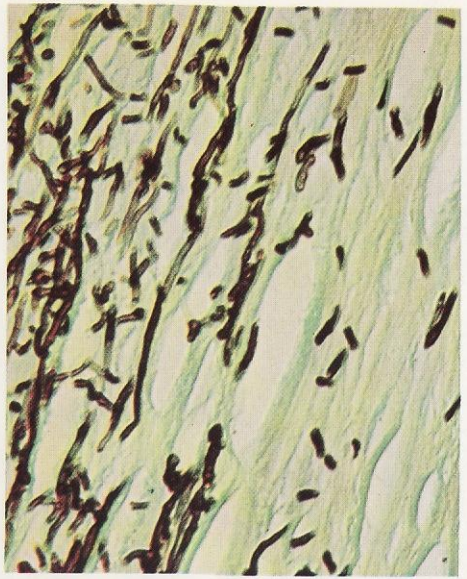
Contusion of eye by tree branch, corneal ulcer due to fungus. C. Gridley fungus stain: fungi—red; background—yellow. D. Periodic acid-Schiff stain (PAS) without counterstain: fungi—magenta. E. Gomori's methenamine silver stain (GMS): fungi—black; background—light green. (From AFIP Exhibit No. 907. Accession No. 831164.)



B



C



E

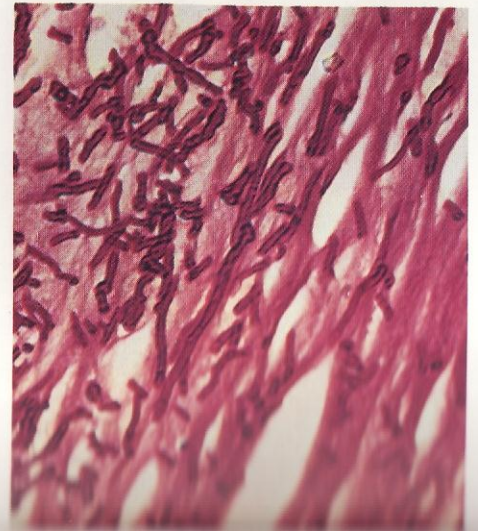
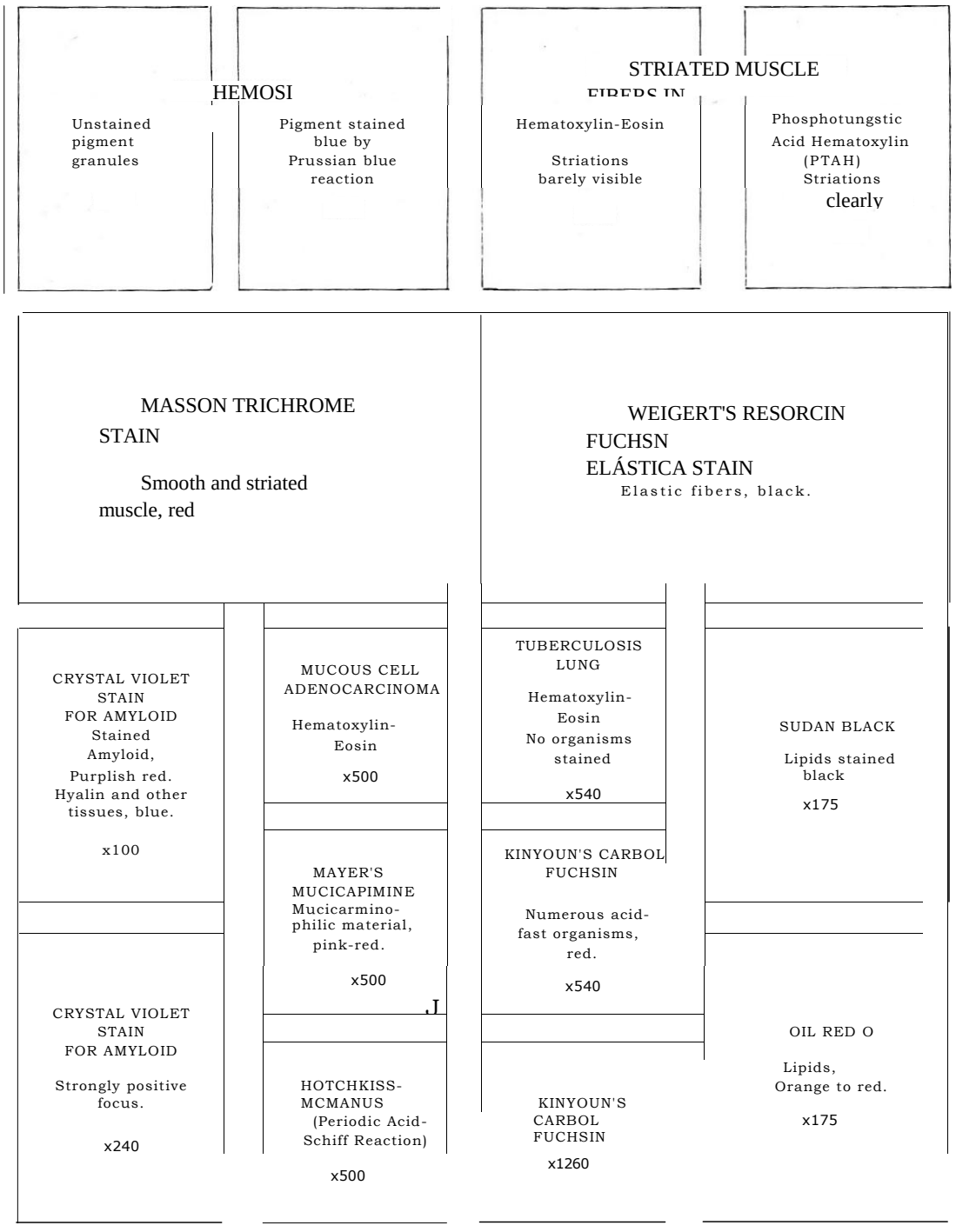


PLATE II



Reprinted froco J. E. Ash and M. Raum: An atlas of Otolaryngic Pathology, American Registry of Pathology. 1956.

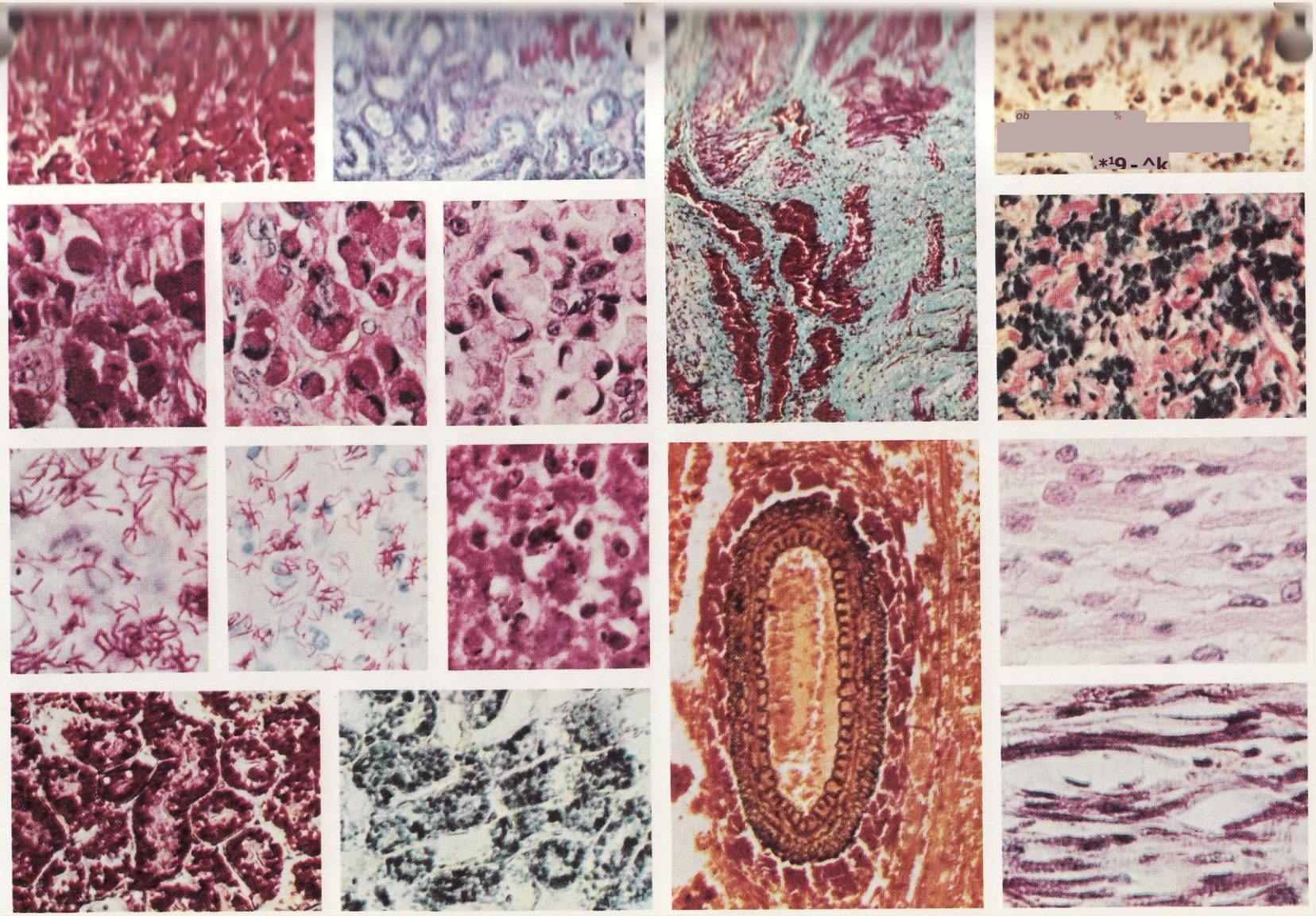
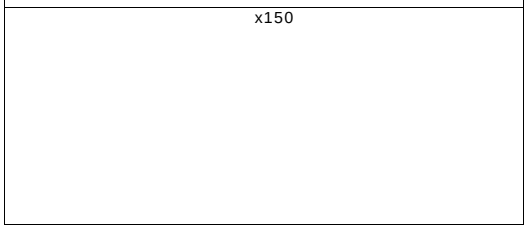


PLATE 111

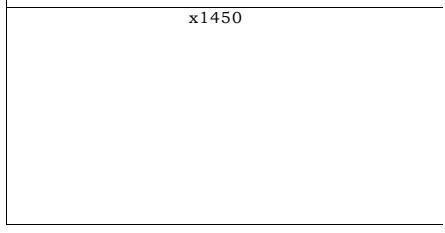
Encapsulated Cryptococci Demonstrated by Special Staining Procedures

Hematoxylin-Eosin



x150

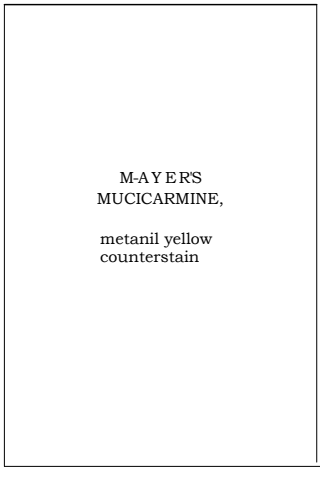
Toluidipe blue,
frozen section.



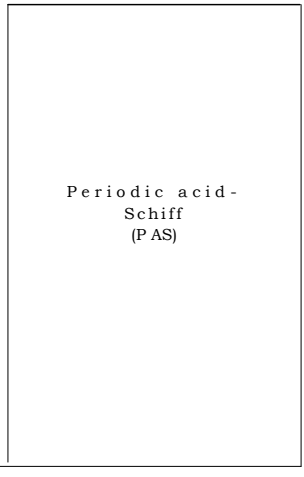
x1450

M-AYER'S
MUCICARMINE,

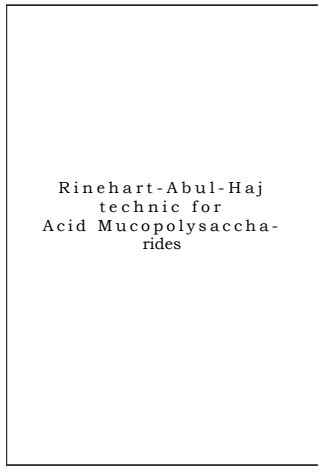
metanil yellow
counterstain



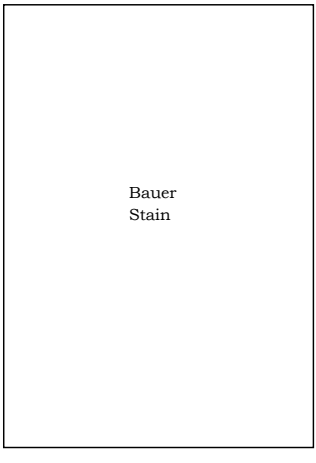
Periodic acid-
Schiff
(PAS)



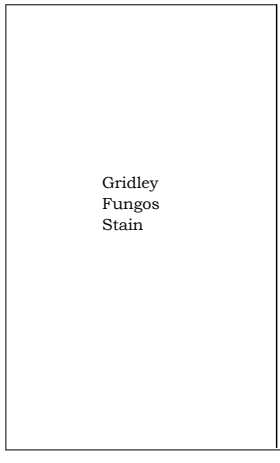
Rinehart-Abul-Haj
technic for
Acid Mucopolysaccha-
rides



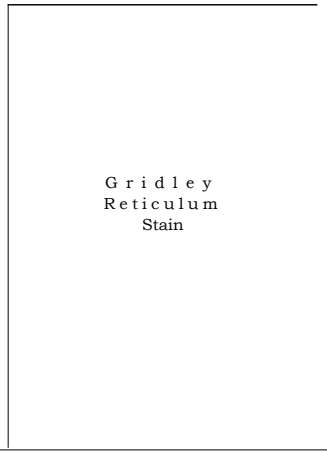
Bauer
Stain



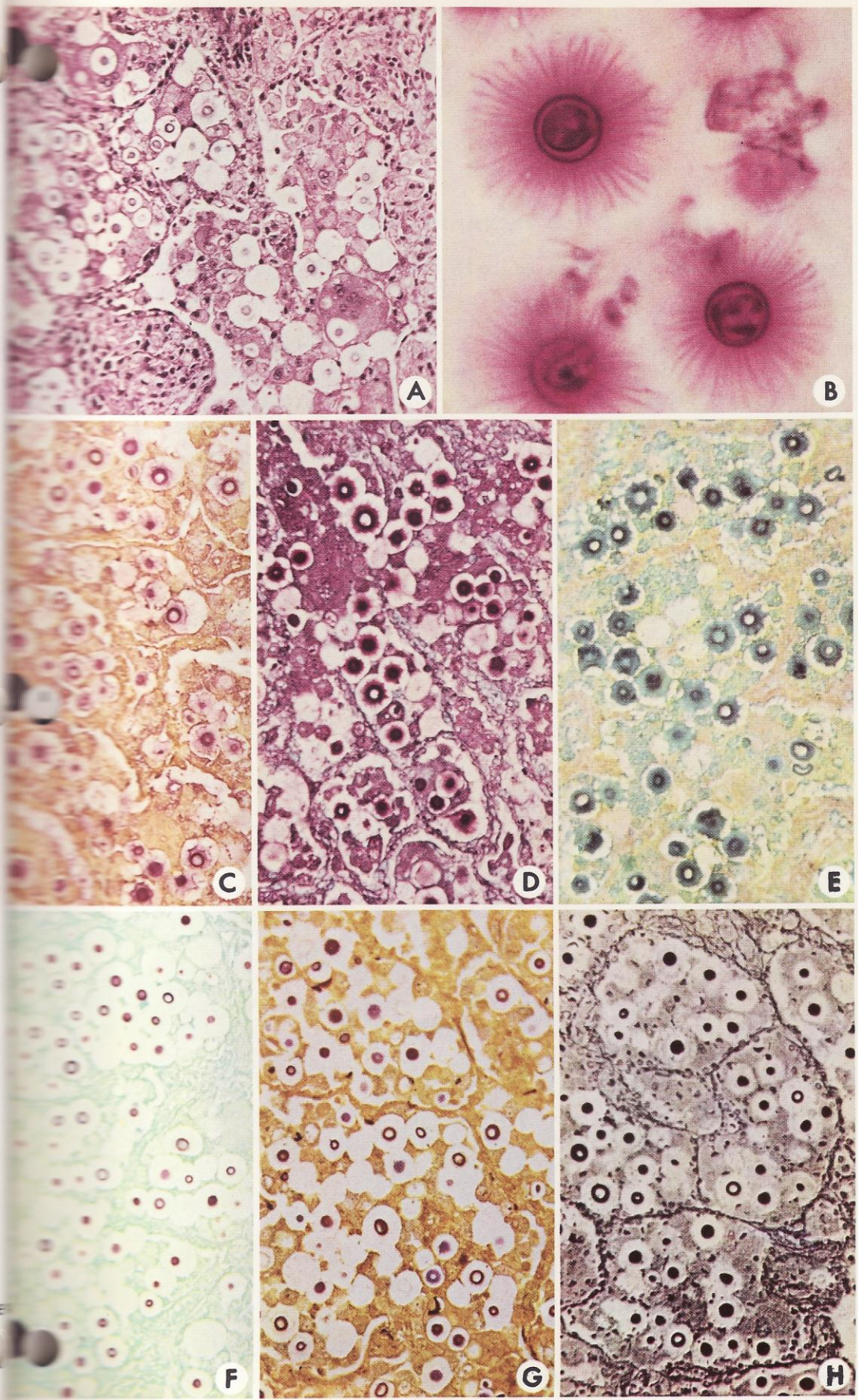
Gridley
Fungos
Stain



Gridley
Reticulum
Stain



Reprinted froco M. L. Littman and L. E. Zimmerman: Cryptococcosis, 1
York and London, Grune and Stratton, 1956.


A
B
C
D
E
F
G
H

REFERENCE. 1. Weibel, E., Shenk, R. Morger, R., and Toendury, G.: *The preparation of serial microscopic sections in forco of plastic films. 2.* Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C. *20305.*

MULTIPLE STAINING ON ONE SLIDE

The following technic is useful when one desires multiple special stained sections on one slide.

STAINING PROCEDURE

1. Deparaffinize in three changes of xylene.
2. Cover sections *not* to be stained with: Dow Corning 7 Compound* (xylene must evaporate froco section before compound is applied), *or* Diatex + (while xylene damp). Permit datei to harden *30* minutes.
3. Dip in 80% alcohol and distilled water.
4. Perform stain desired. Only the uncovered section will stain.
5. Placo in xylene, several changes, until covering media is completely dissolved.
6. Repeat Steps *2* thru *5* for as many stains required or number of sections available.

Figure 19 shows *one* slide as it undergoes application of Dow Corning or Diatex

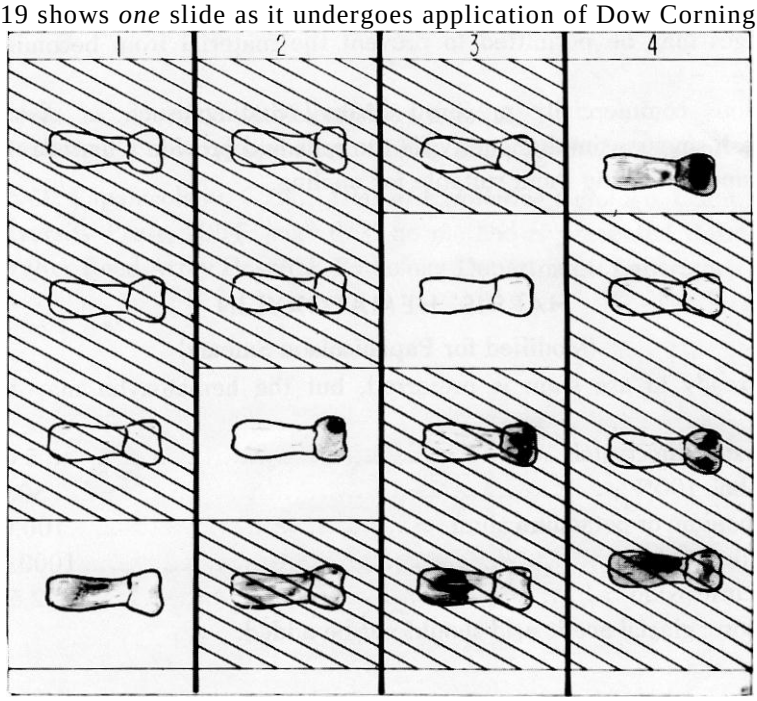


Fig. 19. Plan for multiple staining on one slide.

*Dow Corning 7 Compound, Dow Corning Company, Midland, Michigan. 07605 Diatex Mounting Media, Scientific Products Inc., 2020 Ridge Ave., Evanston, 111. 60201

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C. 20305.

PAPANICOLAOU METHOD OF STAINING SMEARS

PREPARATION OF SMEARS

1. Exfoliated cells degenerate rapidly; therefore smears should be prepared promptly and fixed immediately. If there is to be any delay, the specimen should be fixed in 95% alcohol and refrigerated until smears can be prepared. Specimens requiring centrifugation (e.g., urine and ascitic fluid) are preserved by adding an equal volume of 50% ethyl alcohol; centrifugation is at 2000 rpm for 30 minutes.
2. Viscid secretions (e.g., vaginal, cervical and prostatic) should be smeared directly onto clean glass slides and fixed immediately.
3. Body fluida and watery exudates (e.g., urine, spinal fluid, pleural fluid, etc.) will not properly adhere to the glass slides unless the slide is first coated with a layer of Mayer's egg albumin (one drop per slide).
4. The sediment of centrifuged specimens is smeared onto glass slides coated with MayeVs egg albumin. Any remaining sediment should be processed as a biopsy specimen for conventional histologic examination.

FIXATION

1. Alcohol, 95% -15 minutes
2. Smears should be fixed immediately while still wet, though partial drying along the edges may be permitted to prevent the material from becoming detached from the slide.
3. Various commercially prepared fixative coatings such as cytospray when applied to fresh smears, simultaneously fix the cells and provido a hard protective coating over the smear, making them suitable for mailing.

SOLUTIONS

HARRIS' HEMATOXYLIN

(Modified for Papanicolaou Smears)

Ortho's ready to use stain is preferred, but the hematoxylin may be prepared as follows:

Hematoxylin crystals	5.0 gm
Alcohol, 100%	50.0 ml
Ammonium or potassium alum	100.0 gm
Distilled water	1000.0 ml
Mercuric oxide	2.5 gm

For this purpose, glacial acetic acid should *not* be added.

ORANGE G6 (OG 6)

Ortho's* ready to use stain is preferred but the stain may be prepared as follows:

Orange G6, 0.5% solution in 95% alcohol	100.0 ml
Phosphotungstic acid	0.015 gm

[0] Ortho Diagnostica, Raritan, New Jersey, 18869

EOSIN-AZURE 50 (EA 50)

This stain should be obtained from the Ortho Diagnostica, Raritan, New Jersey. It is ready for use after it is filtered and cannot be equalled by mixtures prepared in the laboratory.

STAINING PROCEDURE

1. After fixation, transfer slides without drying directly from 95% alcohol through 501% alcohol, 70% alcohol, 50% alcohol and tap water, to distilled water.
2. Stain in Harris' hematoxylin using either (a) Papanicolaou's modified hematoxylin (Ortho), 2 to 3 minutes, or (b) Harris' alum hematoxylin without glacial acetic acid, 2 to 3 minutes.
3. Rinse gently in tap water.
4. Differentiate in 0.25% hydrochloric acid in distilled water, 1 or 2 dips.
5. Place in gently running tap water for 5 minutes to wash out the acid and thoroughly blue the nuclei.
6. Rinse in distilled water and transfer through 50% alcohol, 70% alcohol, 80% alcohol to 95% alcohol.
7. Stain in OG 6 for 2 minutes.
8. Rinse in three changes of 95% alcohol.
9. Stain in EA 50 for 2 minutes.
10. Rinse in three changes of 95% alcohol, two changes of absolute alcohol and :our changes of xylene.
11. Mount with Permount or balsam.

RESULTS

Nuclei	- blue with clear sharp details
Cytoplasm	- varying shades of pink, blue, yellow, green and gray

REFERENCE. Papanicolaou, G. N.: *Atlas of Exfoliative Cytology,* Cambridge, Mass., -íarvard University Presa, 1954, page 6. (The method is presented through the cour'es~' of the Walter Reed Army Hospital, Pathology Department,

BORATE BUFFER SOLUTION, pH 8.2 (WORKING)

Solution A	6.5 ml
Solution B	3.5 ml

1% GOLD CHLORIDE SOLUTION (STOCK)

(See pape 90)

GOLD CHLORIDE SOLUTION (WORKING)

Gold chloride stock solution	10.0 ml
Distilled water	40.0 ml

Solution is stable for approximately 100 slides.

3% SODIUM THIOSULFATE (HYPO) SOLUTION

Sodium thiosulfate	3.0 gm
Distilled water	100.0 ml

METHENAMINE SILVER SOLUTION, pH 8.2 (WORKING)

Methenamine, 3%	42.5 ml
Silver nitrate, 5%	2.5 ml
Borate buffer, pH 8.2	12.0 ml

Prepare fresh just before use and filter. This solution is stable for approximately 60-75 minutes. After this time, there is a breaking down process, which produces a black precipitate and is picked up on the slides.

STAINING PROCEDURE. Chemically clean glassware must be used.

Note. It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chloride free distilled water. Distilled water may be checked for free chloride by the addition of several drops of 5% silver nitrate solution. If a white cloud appears upon the addition of the silver nitrate, discard the sample of water and replace.

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 11 minutes.
3. Rinse in chloride free distilled water.
4. Filter freshly prepared methenamine-silver solution into coplin jar.
5. Place slides in methenamine-silver solution and then place coplin jar in prewarmed 70 °C water bath. Start timing at this point, approximately 60-75 minutes. Check under microscope when slides show macroscopically a medium brown color.

Note. Solution and slides should be allowed to come to 70 °C together. While slides are in the silver solution they may be examined after they begin to show macroscopically a medium brown color reaction. Before checking under the microscope, they are first rinsed in hot 70 °C chloride free distilled water, checked, and then returned to hot water rinse and then returned into hot staining solution. Slides should be checked every 10 minutes when they have reached the dark or medium brown stage. Slides should be checked as rapidly as possible because if the section cools there is an up

Chapter 6

Methods For Connective Tissue

LIMES ALLOCHROME METHOD FOR CONNECTIVE TISSUE

FIXATION. Any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SCHIFF REAGENT SOLUTION

(See page 159)

0.5% PERIODIC ACID SOLUTION

Periodic acid	0.5 gm
Distilled water	100.0 ml

0.5% SODIUM METABISULFITE SOLUTION

Sodium metabisulfite	0.5 gm
Distilled water	100.0 ml

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

PICRIC ACID-METHYL BLUE SOLUTION

Picric acid, saturated aqueous	100.0 ml
Methyl blue	40.0 mg

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 10 minutes.
3. Wash in tap water for 5 minutes.
4. Schiff's reagent solution for 10 minutes.
5. Sodium metabisulfite solution for two changes, 2 minutes each.
6. Wash in tap water for 10 minutes.
7. Weigert's irou hematoxylin solution for 2 minutes.
8. Wash in tap water for 10 minutes.
9. Picric acid-methyl blue solution for 6 minutes.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Nuclei	- black, gray, or brown
Cytoplasm and Muscle cells	- gray, green to greenish yellow
Reticulum and Collagen	- blue
Basement membranas	-red

REMARKS. Valuable for the distinction between basement membrana and reticulum fibers and the demonstration of arteriolosclerotic lesions.

REFERENCE. Lillie, R. D., *Amer. J. Clin. Path. 21:484-488, 1951.* Copyright by Williams arid-Wilkins Co.

PUCHTLER-SWEAT METHOD FOR BASEMENT MEMBRANES

FIXATION. Carnoy's solution No. 2

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CARNOY'S SOLUTION NO. 2

Alcohol, 100%	60.0 ml
Chloroform	30.0 ml
Glacial acetic acid	10.0 ml

0.5% PERIODIC ACID SOLUTION

(See page *72)*

SODIUM BISULFITE SOLUTION

Sodium bisulfite	20.0 gm
Distilled water	*40.0* ml
Alcohol, 100%	10.0 ml

RESORCIN - FUCHSIN SOLUTION

(See page *79)*

KERNECHTROT SOLUTION

(See page *89)*

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 5 minutes.
3. Rinse in distilled water, two changes.
4. Sodium bisulfite solution for *15* hours.
5. Rinse in distilled water, five or six changes.
6. Resorcin-fuchsin solution for 4 hours.
7. Rinse in distilled water, three changes.
8. Counterstain in Kernechtrot solution for *5* minutes.

10. Dehydrate in *95%* alcohol, absoluta alcohol, and clear in xylene, two changes each.

11. Mount with Permount or Histoclad.

RESULTS

Basement membranas - black in cross sections
gray in tangential. sections
Nuclei - pink to red

REFERENCE. Puchtler, H., and Sweat, F.: *Stain Techn. 39: 163 -166, 1964.*

LUNA-ISHAK METHOD FOR BILE CANALICULI

FIXATION. 10% buffered neutral, formalin is preferred.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CELESTINE BLUE SOLUTION

Celestine blue B ..... 0.5 gm
Ferric ammonium sulfate ..... 5.0 gm
Hydrochloric acid, concentrated ..... 2.0 ml
Glycerin ..... 15.0 MI.
Distilled water .....85.0 **ml**

Dissolve the irou alum in the distilled water, then dissolve the celestina blue in this solution and bring to a boil for 3 -5 minutes. Cool and add the glycerin and hydrochloric acid.

1% PHOSPHOTUNGSTIC ACID SOLUTION

Phosphotungstic acid ..... 1.0 gm
Distilled water ..... 100.0 ml

1% STOCK ACID FUCHSIN SOLUTION

Acid fuchsin .....1.0 gm
Distilled water ..... 100.0 ml

1% STOCK OXALIC ACID SOLUTION

Oxalic acid ..... 1.0 gm
Distilled water ..... 100.0 ml

WORKING ACID FUCHSIN-OXALIC ACID SOLUTION

Acid Fuchsin, stock solution ..... 10.0 ml
Oxalic acid, stock solution ..... 1.0 ml
Distilled water ..... 80.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled. water.
2. Celestine blue solution for 30 minutes.

o

3. Rinse in distilled water.

4. Phosphotungstic acid solution for 15 minutes.
5. Wash in tap water for 3 minutes.
6. Working acid fuchsin solution for 8 minutes

7. Dehydrate rapidly in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

Bile Canaliculi –pink to red
Nuclei –blue
Bile –yellowish - green
I r o u -brown with some red inclusions
Background –bluish - pink alcohol will remove red dye on prolonged exposure, thus the ration.
Note. Water and
reason for rapid dehyd

REFERENCE. Luna, L. G., and Ishak, K.: *Amer. J. Med. Techn.* 33:459-466, 1967.

MALLORY'S METHOD FOR COLLAGEN

a

FIXATION. Zenker's

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

0.5% ACID FUCHSIN SOLUTION

Acid Fuchsin	0.5 gm
Distilled water	100.0 ml

ANILINE BLUE-ORANGE G SOLUTION

Aniline blue, water soluble	0.5 gm
Orange G	2.0 gm
Phosphotungstic acid	1.0 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (hypo) (see page 41).

3. Wash in running water for 10 to 20 minutes.

4. Rinse in distilled water.

RESULTS

Nuclei	red
Collagen fibrils	blue
Ground substance of Cartilage	varying shades of blue
Mucin	varying shades of blue
Amyloid	varying shades of blue
Erythrocytes	yellow
Basophils (beta cell)	- blue
Acidophils (alpha cell)	- red
Chromophobes	- gray
Myelin	- yellow

REMARKS. Not especially useful for Collagen. However, very useful for pituitary cell differentiation.

REFERENCE. Mallory, F. B.: *Stain Techn.* 11:101-102, 1936. Copyright by Williams and Wilkins Co.

VAN GIESON'S METHOD FOR COLLAGEN FIBERS

FIXATION. Any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

VAN **GIESON'S SOLUTION**

Acid fuchsin, 1% aqueous .....2.5 ml
Picric acid, saturated aqueous ..... 97.5 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Weigert's hematoxylin solution for 10 minutes.
3. Wash in distilled water.
4. Van Gieson's solution for 1 to 3 minutes.
5. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Collagen	- red
Muscle and Cornified epithelium	- yellow
Nuclei	- blue to black

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 152.

PINKUS' ACID ORCEIN-GIEMSA METHOD

FIXATION. 10% buffered neutral formalin, formol alcohol, or absolute alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns or 10 microns (See *Note).*

SOLUTIONS

ACID ORCEIN SOLUTION

Orcein, Synthetic Harleco*	0.2 gm
Alcohol, 70%	100.0 ml
Hydrochloric acid, concentrated	0.6 ml

Solution is ready for staining immediately and improves on standing. It is stable for many months.

DILUTE GIEMSA SOLUTION

Giemsa, any good stock solution	1 drop
Distilled water, adjusted to pH 7.0 with a phosphate buffer	20.0 ml

ALCOHOL EOSIN SOLUTION

Eosin, 1% alcoholic	2.0 ml
Alcohol, 95%	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to 70% alcohol.
2. Acid orcein solution for 30 minutes.
3. Wash in running tap water for 15 minutes.
4. Stain overnight in dilute Giemsa solution. Do not hurry this step.
5. Drain off excess fluid. If sections do not have the desired rose-pink color of the collagen, decolorize slightly and counterstain with eosin. Personal preferente and experiente controls this step.
6. Dehydrate in absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Nuclei	- deep blue
Cytoplasm of epidermis, smooth muscle, and other cells	- light blue
collagen	- rose pink
Elastic fibers	- dark brown
Mast cell granules and many mucoid substances	- purple
Eosinophilic granules and erythrocytes	- bright red

* Hartman - LeMen Co., Philadelphia, Pennsylvania 19 142

Note: Sections cut at ten microns are preferable, sinta they give a better three-dimensional. picture. Step 2 stains elastic fibers specifically. If background becomes overstained; decolorize by dipping in absolute alcohol or 0.1 % hydrochloric acid alcohol.

REFERENCE. This stain was modified froco the original Unna-Taenzer procedure by Hermann Pinkus *(Arch. Dermat. & Syph. 49: 355, 1944)* and further modified and simplified by Hermann Pinkus and Rosie Hunter.

GOMORIS ALDEHYDE FUCHSIN METHOD

FIXATION. 10% buffered neutral formalin, or any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALDEHYDE FUCHSIN SOLUTION

Basic fuchsin	1 0 gm
Alcohol, *70%*	200.0 ml
Hydrochloric acid, concentrated	2.0 ml
Paraldehyde	2.0 ml

Let stand at room temperatura for 2 to *3* days or until stain is deep purple in color.

-VAN GIESON'S SOLUTION

..(See paga *76)*

0.25% METANIL YELLOW SOLUTION

Metanil yellow	0 *25* gm
Distilled water	100.0 ml
Glacial acetic acid	0 *25* ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Rinse in several changes of *95%* alcohol.
3. Aldehyde fuchsin solution for 30 minutes.
4. Rinse off excess stain in *95%* alcohol.
5. Rinse in water.
6. Counterstain as desired with Van Gieson's solution, or metanil yellow, or Gomori's trichrome stain.
7. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Elastic fibers and mucin - Deep purple

Other tissues are stained according to the counterstain used.

REMARKS. Aldehyde fuchsin has been used quite successfully in the study of mucosaccharides. The details for these methods may be found in Chapter 10.

REFERENCE. Gomori, G.: Techn. Bull. Reg. Med. Techn. *7:115 -117, 1946.*

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HART'S METHOD FOR ELASTIC FIBERS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

0.25% POTASSIUM PERMANGANATE SOLUTION

Potassium permanganate	0.25 gm
Distilled water	100.0 ml

5% OXALIC ACID SOLUTION

Oxalic acid	5.Ogm
Distilled water	100.0 ml

RESORCIN-FUCHSIN SOLUTION (STOCK)

Basic fuchsin	2.0 gm
Resorcinol	4.O gm
Distilled water À	200.0 ml

Mix in a procelain dish, bring to a boil and let boil for 1 minute, then add 25 ml of 29% ferric chloride solution. Cool, filter, and leave precipitate on filter paper until dry. Return to porcelain dish, which should be dry but still contains whatever part of the precipitate remains adherent to it. Add 200 ml of 95% alcohol and heat very carefully dissolving all precipitate froco filter paper before discarding it. Add 4 ml of hydrochloric acid. This solution keeps for several months.

RESORCIN - FUCHSIN SOLUTION (WORKING)

Resorcin-fuchsin, stock	10.0 ml
Alcohol, 70%	100.0 ml
Hydrochloric acid, concentrated	2.0 ml

VAN GIESON'S SOLUTION

(See page 76)

STAINING **PROCEDURE**

1. Deparaffinize and hydrate to distilled water.
2. Potassium permanganate solution for 5 minutes.
3. Rinse in distilled water.
4. Oxalic acid solution until sections are clear.
5. Wash in tap water for 5 minutes then place in distilled water.
6. Working solution of resorcin-fuchsin overnight.
7. Wash in tap water for 10 minutes.
8. Rinse in distilled water.
9. Van Gieson solution for 1 minute.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes ~~~~h.
11. Mount with Permount or Histoclad.

RESULTS

Elastic fibers	-blue-black to black
Nuclei	-blue to black
Collagen	-pink to red
Other tissue elements	-yellow

REMARKS. This method is especially useful in the demonstration of fine elastic fibers and elacin (degenerated elastic fibers).

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 169. AFIP modification.

WEIGERT'S METHOD FOR ELASTIC FIBERS

FIXATION. Any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION
(See page 35)

RESORCIN-FUCHSIN SOLUTION
(See page 79)

VAN GIESON'S SOLUTION
(See page 76)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Weigert's hematoxylin solution for 10 minutes.
3. Wash well in water. Intensifies stain.
4. Resorcin-fuchsin solution for 30 minutes or longer.
5. Rinse in 95% alcohol.
6. Wash in tap water.
7. Van Gieson's solution for 1 minute. Save solution. If overstained, water will remove excess van Gieson's solution.
8. Dehydrate in 95% alcohol, absolute alcohol, and olear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Elastic fibers	-blue-black to black
Nuclei	blue to black
Collagen	pink to red
Other tissue elements	yellow

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p.168.

FRASER-LENDRUM METHOD FOR FIBRILA

FIXATION. Zenker's.

TECHNIQUE. Cut paraffin section at 6 microns.

SOLUTIONS

CELESTINE BLUE SOLUTION

Ferric ammonium sulfate ..... 2.5 gm
Distilled water.....50.0 ml
Dissolve overnight at room temperatura. Add:
Celestina blue .....0.25 gm
Boil for 3 minutes. Filter when cool, add 7 ml glycerin.

MAYER'S HEMATOXYLIN SOLUTION

(S e e p a g a 3 3)

ORANGE G-PICRIC ACID SOLUTION

Saturated picric acid in 80% alcohol .....200.0 ml
Orange G ..... 0.4 gm

1% ACID FUCHSIN SOLUTION

Acid fuchsin ..... 1.0 gm
Distilled water .....99.0 ml
Glacial acetic acid .....1.0 ml

DIFFERENTIATING SOLUTION

Orange G - Picric acid solution .....30.0 ml
Alcohol, 80% .....70.0 ml

MACFARLANE'S SOLUTION (STOCK)

Phosphotungstic acid ..... 25.0 gm
Picric acid ..... 2.5 gm
Alcohol, 95% .....100.0 ml

MACFARLANE'S SOLUTION (WORKING)

MacFarlane's solution, stock.....40.0 ml
Alcohol, 95% .....40.0 ml
Distilled water .....20.0 ml

2% LIGHT GREEN SOLUTION

Light green, SF yellowish ..... 2.0 gm
Distilled water .....98.0 ml
Glacial acetic acid ..... 1.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Mordant in Zenker's solution overnight if formalin fixed.
3. Wash in running water.

4. Remove mercuric chloride crystal with iodine and clear with sodium thiosulfate (see page 41).

5. Wash in running tap water for *15* minutes.

6. Celestin blue solution for *5* minutes.

7. Wash in tap water.

8. Mayer's hematoxylin solution for *5* minutes.

9. Wash in tap water for *5* minutes.

10. Orange G-picric acid solution for *5* minutes.

11. Wash in tap water for 1 minute.

12. Acid fuchsin solution for *5* minutes.

13. Wash in tap water.

14. Differentiating solution for *10-15* seconds.

15. Wash in tap water.

16. MacFarlane's working solution for *5* minutes.

17. Wash in tap water.

18. Counterstain in light green solution for 1 minute.

19. Wash in tap water.

20. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes each.

21. Mount with Permount or Histoclad.

ANILINE BLUE-ORANGE G SOLUTION

(See page 75)

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STAINING PROCEDURE. For consistent staining results *use fresh solutions.*

1. Deparaffinize and hydrate to distilled water.
2. Acid fuchsin solution for 3 minutes.
3. Transfer slides directly to aniline blue-orange G solution for 45 minutes.
4. Transfer slides directly to 95 /"/c alcohol for three changes.
5. Dehydrate with absolute alcohol and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Keratin	- brilhant red
Stratified squamous epithelium	- gray
Stratum spinosum, upper area illustrating pre-keratin-like substances	- orange
Connective tissue	- deepblue
Erythrocytes	- deepred

REMARKS. This is a modification of Mallory's connective tissue stain.

REFERENCE. Ayoub, P., and Shklar, G.: *J. Oral Surg.* 16:580 - 581, 1963. Copyright by American Dental Association. (Reprinted by permission).

DANES METHOD FOR PREKERATIN, KERATIN, AND MUCIN

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffiri sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

1% PHLOXINE SOLUTION

Phloxine ..... 1.0 gm
Distilled water ..... 100.0 MI.

ALCIAN **BLUE** SOLUTION

Alcian blue, 1/07 6 ..... 50.0 ml
Glacial acetic acid , I/ll 6 ..... 50.0 ml

ORANGE G SOLUTION

Orange G ..... -0.5 gm
Phosphotungstic acid ..... . 2.0 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 10 minutes.
3. Blue in running tap water for 10 minutes, then rinse in distilled water.
4. Phloxine solution for 3 minutes
5. Wash in running tap water to remove excess phloxine.
6. Rinse in distilled water.
7. Alcian blue solution for 5 minutes.
8. Wash in tap water for 2 minutes, then rinse in distilled water.
9. Orange G solution for 13 minutes.
10. Transfer slides to 95/^{Ol}o alcohol, two changes, five dips each.
11. Absoluto alcohol, two changes, 15 dips each.
12. Clear in xylene.
13. Mount with Permount or Histoclad.

RESULTS

Acid mucopolysaccharides	turquoise blue
Prekeratin and keratin	orange to red-orange
Nuclei	orange to brown

REFERENCE. Dane, E. T., and Herman, D. *L.: Stain Techn.* 38:97-101, 1963.
Copyright by Williams and Wilkins Co.

MENZIES' METHOD FOR MUSCLE STRIATIONS

FIXATION. 10% buffered neutral formalin

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BROMPHENOL BLUE SOLUTION

Bromphenol blue"	0.05 gm
Distilled water	100.0 ml
Glacial acetic acid	2.0 ml

0.5% ACETIC ACID SOLUTION

Glacial acetic acid	0.5 ml
Distilled water	100.0 ml

SCOTT'S WATER SOLUTION

Sodium or potassium bicarbonato	2.0 gm
Magnesium sulfate	20.0 gm
Distilled water	1000.0 ml

Dissolve separately and then combine. A few crystals of thymol or 5 to 10 ml of formaldehyde (40%) solution is added to prevent the growth of mold.

*Tetrabromophenolsulphonphthalein

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Bromphenol blue solution 4 to 24 hours.
3. Rinse briefly in water and placa in acetic acid solution for *5* to 10 minutes.
4. Wash and blue in Scott's water solution for 30 seconds.
5. Rinse in distilled water.

6. Dehydrate in *95%* alcohol, absoluta alcohol, and clear in xylene, two changes each.

7. Mount with Permount or Histoclad.

RESULTS

Cardiac and skeletal muscle - intensa blue

Due to the densa background, cardiac and skeletal muscle striations require oil immersion for full resolution.

Note. Scott's water solution used for blueing also differentiates by removing excess dye, but the 30 second treatment should not be exceeded. Immersion for 5 to 10 minutes in acetic acid solution between the primary stain and the Scott's water solution has a definita effect in heightening contrast.

REFERENCE. Menzies, D. W.: ***Stain Techn. 36:285 -287,1961.***

Copyright by Williams and Wilkins Co.

MALLORY'S PHOSPHOTUNGSTIC ACID HEMATOXYLIN METHOD (PTAH)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at *6* microns.

SOLUTIONS

PHOSPHOTUNGSTIC ACID HEMATOXYLIN SOLUTION

Hematoxylin	1.0 gm
Phosphotungstic acid	*20.0* gm
Distilled water	1000.0 ml

Dissolve the solid ingrediente in separata portions of the water, the hematoxylin with the aid of heat. When cool, combine. No preservativa is necessary. Ripen by the addition of 0.2 gm potassium permanganate. Stain may be used immediately but the best resulte are not obtained until the solution is at least two weeks old.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Mordant in Zenker's solution that contains *5%* acetic acid for 3-1/2 hours in a 56 ° - 60 °C oven

8. Dehydrate rapidly in *95%* alcohol, absoluto alcohol, and clear in xylene, two changes each.

9. Mount with Permount or Histoclad.

RESULTS

Muscle	- blue, with cross striations well defined
Collagen	- red
Nuclei	blue
Fibrin	- blue

REMARKS. Excellent technic for all types of tissue (Exception: Central Nervous System, soe Chapter *12).*

Note. Modifications by Lee G. Randle, HMCS, USN, AFIP. The use of potassium permanganate is an excellent oxidizing media sinto the PTAH solution can be used immediately. More important is the fact that the staining is more consistent when oxidized in this manner. At step 2 Zenkerization of slides in oven for 3-1/2 hours has stabilized the consistency of the staining results. At step 7, 90 minutes **in** the oven is necessary for complete staining.

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C. *20305.*

HEIDENHAIN'S ANILINE BLUE METHOD

FIXATION. Zenker's, Helly's, Bouin's, Carnoy's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% AZOCARMINE B SOLUTION

Azocarmine B	1.0 gm
Distilled water	100.0 ml

Bring to a boil, filter at *56* ° C, cool and add 1 ml glacial acetic acid. Must be kept in refrigerator and filtered before use.

1% ANILINE - ALCOHOL SOLUTION

Aniline	1.0 ml
Alcohol, *95%*	100.0 ml

5% PHOSPHOTUNGSTIC ACID SOLUTION

Phosphotungstic acid	5.0 gm
Distilled water	100.0 ml

ANILINE BLUE SOLUTION

Aniline blue, water soluble	0.5 gm
Orange G	*2.0* gm

Distilled water .....300.0 ml
Glacial acetic acid ..... 8.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled 'water.

2. If formalin fixed, mordant in Zenker's overnight, then remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).

3. Wash in running tap water.

4. Rinse in distilled water.

5. Place in preheated azocarmine B solution at 56 'C for 15 minutes.

6. Rinse in distilled water.

7. Differentiate in aniline-alcohol solution until cytoplasm and connective tissue are pale pink and nuclei stand out sharply. (Control differentiation by rinsing in 1% acetic alcohol).
8. Mordant in phosphotungstic acid solution for 15 minutes.
9. Rinse in distilled water.
10. Aniline blue solution for 15 minutes.
11. Rinse in distilled water.

12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes, 2 minutes each.

13. Mount with Permount or Histoclad.

RESULTS

Chromatin, osteocytes, neuroglia	- red
Collagen, reticulum	- blue
Muscle	- red to yellow
Osteoid material in decalcified sections	- red

REFERENCE. Mallory, E B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 154 -155.

GOMORVS METHOD FOR RETICULUM

FIXATION 10% buffered neutral formalin. TECHNIQUE.

Cut paraffin sections at 6 microns.

SOLUTIONS

2% POTASSIUM METABISULFITE SOLUTION

Potassium metabisulfite ..... 2.0 gm
Distilled water ..... 100.0 ml

2% FERRIC AMMONIUM SULFATE SOLUTION

Ferric ammonium sulfate ..... 2.0 gm
Distilled water ..... 100.0 ml

20% FORMALIN SOLUTION

Formaldehyde, 37-40% ..... 20.0 ml
Distilled water ..... 80.0 ml

0.2% GOLD CHLORIDE SOLUTION

(See page 92)

2% SODIUM THIOSULFATE SOLUTION

Sodium thiosulfate .... 2.0 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Oxidize in potassium permanganate solution for 1 minute.
3. Wash in tap water 2 minutes.
4. Differentiate with potassium metabisulfite solution for 1 minute.
5. Wash in tap water for 2 minutes.
6. Sensitize in, ferric ammonium sulfate solution for 1 minute.

7. Wash in tap water for 2 minutes; follow with two changes of distilled water 30 seconds each.

8. Impregnate in the silver solution for 1 minute.

9. Rinse in distilled water for 20 seconds.

10. Reduce in formalin solution for 3 minutes.

11. Wash in tap water for 3 minutes.

12. Tone in gold chloride solution for 10 minutes.

MANUELS METHOD FOR RETICULUM

FIXATION. I0l[t/7]o buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% URANIUM NITRATE SOLUTION

Uranium nitrate ..... 1.0 gm
Distilled water ..... 100.0 ml

AMMONIACAL SILVER SOLUTION

In 1000 ml of distilled water dissolve 100 gm of silver nitrate. Pour off 70 ml and save. To the 930 ml of 10% silver nitrate add 60 ml of 28% ammonium hydroxide, shaking the flask vigorously (solution becomes dark then clears). Of the 70 ml saved, add slowly 50 ml to the ammonium hydroxide silver nitrate mixture. Solution should become slightly cloudy, if not, add part or all of the remaining 20 ml of the silver nitrate. Let stand overnight and filter bafore using. Store in the refrigerator and use as needed.

1% FORMALIN SOLUTION

(See page 90)

1% GOLD CHLORIDE SOLUTION

(See page 90)

5% SODIUM THIOSULFATE (HYPO) SOLUTION

Sodium thiosulfate ..... 5.0 gm
Distilled water ..... 100.0 ml

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

Dissolve 0.1 gm nuclear fast red in 100 ml of 5% solution of aluminum sulfate with aid of heat. Cool, filter, add grain of thymol as a preservativa.

STAINING PROCEDURE

1. **Deparaffinize and hydrate to distilled water.**
2. Sensitize in uranium nitrate solution for 2 minutes.
3. *Dip quickly in running water.*
4. Impregnate in ammoniacal silver solution for 1 minute.
5. **Wash in running water, two or three quick dips (until no more**

12. Counterstain with Kernechtrot solution for 5 minutes.
13. Rinse in distilled water for three changes.
14. Dehydrate in 95% alcohol, absoluto alcohol, and clear in, xylene, two changes each.
15. Mount with Permount or Histoclad.

RESU LTS

Reticulum	- black
Nuclei and background	- red

REMARKS. Originally, the ammoniacal silver used in this technique was employed by Fontana and Masson to demonstrate argentaffin granules.

REFERENCE. Stain developed and to be published by Mr. Benedicto Manuel, Jr. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C. 20305.

SNOOK'S METHOD FOR RETICULUM

FIXATION. 10% buffered formalin.

T EC H N 1 Q U E. Cut paraffin sections at 6 microns.

SOLUTIONS

0.25% POTASSIUM PERMANGANATE SOLUTION

(See page 79)

5% OXALIC ACID SOLUTION

(See page 79)

1% URANIUM NITRATE SOLUTION

(See page 89)

1% FORMALIN SOLUTION

Formaldehyde, 37 –40/$^{0;}_{6}$	1.0 ml
Distilled water	100.0 ml

1% GOLD CHLORIDE SOLUTION

Gold chloride	1.0 gm
Distilled water	100.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

5% SILVER NITRATE SOLUTION

Silver nitrate ..... 5.0 gm.
Distilled water ..... 100.0 ml

10% SODIUM HYDROXIDE SOLUTION

Sodium hydroxide ..... 10.0 gm,
Distilled water ..... 100.0 ml

AMMONIACAL SILVER SOLUTION

To 20 ml of 5% silver nitrate solution in an acid-clean graduated glass cylinder, add 20 drops of 10% sodium hydroxide solution. Then add ammonium hydroxide, drop by drop, until only a few granules of the resulting precipitate remain on the bottom of the cylinder. Add distilled water to make 60 ml, filter and use at once. The ammonium hydroxide must be fresh.

STAINING PROCEDURE. Use acid-clean coplin jars and paraffin-coated forceps for steps 8 through 18. Replace solutions after 10 or 12 slides have been processed, one slide at a time.

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in potassium permanganate solution for 5 minutes.
3. Wash in tap water.
4. Oxalic acid solution until sections are clear.
5. Wash in tap water, then place in distilled water.
6. Mordant in uranium nitrate solution for 5 seconds.
7. Wash in running water.
8. Ammoniacal silver solution for 1 minute.
9. Dip in running water.
10. 11¹˙⁷o formalin solution for 1 minute.
11. Wash in running water.
12. Gold chloride solution until section is grayish black (1 minute).
13. Wahs in running water.
14. Sodium, thiosulfate solution (hypo) 30 seconds to 1 minute.
15. Wash in running water.
16. Counterstain (if desired) in nuclear fast red solution for 5 minutes.
17. Rinse in distilled water.
18. Dehydrate through 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
19. Mount with Permount or Histoclad.

RESULTS

Reticulum, fibers -gray to black
Background - pink to rose

REMARKS. See Wilder's Reticulum, Method

REFERENCE. Snook, *T.: Anat. Rec. 89* :413 -427, 1944.

WILDER'S METHOD FOR RETICULUM

FIXATION. 10% buffered neutral formalin.

TEC H N 1 QU E. Cut paraffin sections at 6 microns.

SOLUTIONS

10% PHOSPHOMOLYBDIC ACID SOLUTION

Phosphomolybdic acid ..... 10.0 gm
Distilled water .....100.0 ml

1% URANIUM NITRATE SOLUTION

(S e e p a g e 8 9)

AMMONIACAL SILVER SOLUTION

To 5 ml of 10.2% aqueous solution of silver nitrate add 28% ammonium hydroxide, drop by drop, until the precipitate which forros is almost dissolved. Add 5 ml of 3.19,0 sodium hydroxide and barely dissolve the resulting precipitate with a few drops of ammonium hydroxide. Make the solution up to 50 ml with distilled water. Use at once. Glassware must be acid-clean.

REDUCING SOLUTION

Distilled water .....50.0 ml
Neutral formalin, 401`6 ..... 0.5 ml
Uranium nitrate, I % aqueous solution ..... 1.5 ml

Make fresh just before use.-'Calcium carbonate in excess.

0.2% GOLD CHLORIDE SOLUTION

Gold chloride solution, 1 1 10 ..... 10.0 ml

Break glass vial (15 grains) in graduated cylinder with 100.0 ml distilled water for 1% solution.

Distilled water .....40.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in phosphomolybdic acid solution for 1 minute.
3. Rinse well in running water or cells will hold yellow.
4. Sensitize in uranium nitrate solution for 1 minute.
5. Rinse in distilled water for 10 to 20 seconds.
6. Ammoniacal silver solution for 1 minute (change solution frequently).
7. Dip very quickly in 95% alcohol and go immediately finto:
8. Reducing solution for 1 minute (change solution frequently).
9. Rinse well in distilled water.

10. Tone in gold chloride solution for 1 minute or until sections lose their yellow color and turn lavender. Too much toning will make sections red. Check individually under microscope.
11. Rinse in distilled water.
12. Sodium thiosulfate solution for 1 minute.
13. Wash well _ri tap water.
14. Counterstain, if desired, with nuclear fast red solution. Rinse well in distilled water.
15. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes each.
16. Mount with Permount or Histoclad.

RESULTS

Reticulum fibers - black
Collagen - rose
Other tissue elements - red

REMARKS. Well stained reticulum exhibit black, well defined fibers without beading effect and relatively clear background. If beading effect and dark background is noticed replace ammoniacal silver and reducing solution.

REFERENCE. Wilder, *H. C.: Amer. J. Path.* 11:817 -821, *1935.*

GOMORIS ONE STEP TRICHROME METHOD

e

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BOUIN'S SOLUTION

(See page *5)*

TRICHROME STAIN

Chromotrope 2R	0.6 gm
Light green, SF yellowish	*0.3* gm
Glacial acetic acid	1.0 ml
Phosphotungstic acid	0.8 gm
Distilled water	100.0 ml

Aniline blue may be substituted for light green if it is more desirable to have collagen stained blue.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Place in Bouin's solution in oven at 56 °C for one hour.
3. Wash well m running water or until yellow color disappears.
4. Stain nuclei with Weigert's irou hematoxylin or Gomori's chromium hematoxylin solution 10 minutes.
5. Wash in water.

6. Trichrome stain for *15* to 20 minutes.

7. Placo in *0.5%* glacial acetic water for *2* minutes. If sections are too dark, differentiate in 1% glacial acetic water to which 0.7 gm of phosphotungstic acid has been added. Rinse in distilled water.

8. Dehydrate in *95%* alcohol, absoluto alcohol, and elear in xylene, two changes each.

9. Mount with Permount or Histoclad.

RESULTS

Muscle fibers	- red
Collagen	-green (or blue if andino blue is used).
Nuclei	- blue to black

REFERENCE. *Gomori, G.: Amer. J. Clin. Path. 20:661-664, 1950.* Copyright by Williams and Wilkins Co.

MASSONS TRICHROME METHOD

FIXATION. Bouin's or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at *6* microns. SOLUTIONS

BOUIN'S SOLUTION

(See page *5)*

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page *35)*

BIEBRICH SCARLET-ACID FUCHSIN SOLUTION

Biebrich scarlet, aqueous, 1 %	*90.0 ml*
Acid fuchsin, aqueous, 1%	10.0 *ml*
Glacial acetic acid	1.0 *ml*

PHOSPHOMOLYBDIC-PHOSPHOTUNGSTIC ACID SOLUTION

Phosphomolybdic acid	5.0 gm
Phosphotungstic acid	5.0 gm
Distilled water	200.0 ml

ANILINE BLUE SOLUTION

Andino blue	2.5 gm
Glacial acetic acid	2.0 ml
Distilled water	100.0 ml

2% LIGHT GREEN SOLUTION

(See page 81)

1% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Mordant in Bouin's solution for 1 hour at 56 °C, or overnight at room temperature, if formalin fixed.

3. Cool and wash in running water until yellow color disappears.

4. Rinse in distilled water.

5. Weigert's irou hematoxylin solution for 10 minutes. Wash in running water 10 minutes.

6. Rinse in distilled water.

7. Biebrich scarlet-acid fuchsin solution for 2 minutes. Save solution.

8. Rinse in distilled water.

9. Phosphomolybdic-phosphotungstic acid solution for 10 to 15 minutes before aniline blue solution. (Aqueous phosphotungstic acid 5% for 15 minutes before light green counterstain). Discard solution.

10. Aniline blue solution for 5 minutes or light green solution for 1 minute. (for central nervous system 15 to 20 minutes) Save solution.

11. Rinse in distilled water.

12. Glacial acetic solution for 3 to 5 minutes. Discard solution.

13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

14. Mount with Permount or Histoclad.

RESULTS

Nuclei	- black
Cytoplasm, Keratin, Muscle fibers and Intercellular fibers	- red
Collagen	- blue

Note. To insure proper mordanting Bouin's solution should be preheated to 580-600c

WOODSTAIN SCARLET-ACID FUCHSIN SOLUTION

Stock Solution A

Woodstain Scarlet, N. S. concentrate t ..... 0.1 gm
Distilled water .....99.5 ml
Glacial acetic acid..... 0.5 ml

Stock Solution B

Acid fuchsin ..... 0.1 gm
Distilled water .....99.5 ml
Glacial acetic acid .....0.5 ml

Working Solution

Mix 8 parts of Solution A with 2 parts of Solution B.

ALCOHOLIC SAFRAN SOLUTION

Safran du Gatinaist ..... 6.0 gm
Alcohol, 100% .....100.0 ml

After preparing the solution, place in a tightly corked container to prevent hydration and place in an incubator at 58 °C for 48 hours before use. This is done to extract the safran. Keep the solution in an air-tight dark bottle.

STAINING PROCEDURE

1. Deparaffinize and hydrate to water.
2. Rinse thoroughly in distilled water.
3. Alcian blue solution for 20 minutes.
4. Wash in running water for 3 minutes.
5. Alkaline alcohol (ph over 8) for 2 hours. (Prepared by adding 10 ml ammonium hydroxide to 90 ml 95% alcohol. This converts the alcian blue finto the insoluble pigment monastral fast blue).
6. Wash in running water for 10 minutes.
7. Rinse in 70% alcohol.
8. Resorcin-Fuchsin solution for 16 hours.
9. Wash in running water for 10 minutes.
10. Rinse in distilled water.
11. Weigert's hematoxylin solution for 15 minutes.
12. Rinse in running water.
13. Rinse in distilled water.
14. Woodstain scarlet-acid fuchsin solution for 5 minutes. Differentiation of nuclei will take place in this solution.
15. Rinse in 0.5% aqueous glacial acetic acid.
16. Differentiate in 5% aqueous phosphotungstic acid solution for 10-20 minutes, until the collagen is pale pink and the background substance, which is covered by the red, is bluish again.
17. Rinse in 0.5% aqueous glacial acetic acid.

t E. I. duPont de Nemours and Co., Inc., Wilmington, Del. 19898

18. Rinse thoroughly in three changes of absolute alcohol. It is essential not to use low grade alcohols. If low grade alcohols are used, the cytoplasmic stains are dissolved and the tissue will not take on the collagen stain, which is made up in absolute alcohol.

19. Alcoholic safran solution for *5 -15* minutes.

20. Dehydrate in three changes of absolute alcohol, and olear in several changes of xylene. If the collagen is not sufficiently yellow, repeat the staining with safran.

21. Mount with Permount or Histoclad.

RESULTS

Nuclei	- black
Elastic fibers	- dark purple to black
collagen and reticulum fibers	- yellow
Ground substance	- blue to bluish green
Fibrinoid	- intense red
Muscle	- red

REFERENCE. Movat, H. Z.: *Arch. Path., 60 :289 -295, 1955.*

JONES' METHOD FOR KIDNEY

FIXATION. 10% buffered neutral formalin, Bouin's or Zenker's

TECHNIQUE. Cut paraffin sections at *2* microns.

9

SOLUTIONS

0.5% PERIODIC ACID SOLUTION

(See, page *72)*

3% METHENAMINE* SOLUTION

Hexamethylenetetramine (methenamine) ..... *3.0* gm
Distilled water ..... 100.0 ml

5% SILVER NITRATE SOLUTION

(S e e p a g e *9 1)*

BORATE BUFFER SOLUTIONS (STOCK)

Solution A: 0.2 M Boric Acid

Boric acid ..... *12.36* gm
Distilled water .....1000.0 ml

Solution B: 0.25 M Sodium Borate

Sodium borate ..... *19.07* gm
Distilled water .....1000.0 ml

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O Fisher Scientific Co. or Eastman Kodak Co.

even staining of the section. When the desired staining time has been reached, the slide should be checked as described above, every 1- 2 minutes. Strict adherence to the timing is essential in order to obtain a uniform consistency in staining. A properly stained section at this point should have a dark brownish-yellow background; the reticulum fibers will be intense black, as should the basement membranas. An overstained section will be too black. Differentiation will be very difficult as the black will be so intense as to obscura many or all of the tissue elements. The section may be destained with an extremely diluta solution of potassium ferricyanide for one or two dips.

6. Rinse section well in distilled water.

7. Tone in working gold chloride solution for 1 minute.

Note. **If sections are overtoned, placa in 3% sodium metabisulfite for 1-3 minutes, checking periodically.**

8. Rinse well in distilled water.
9. Sodium thiosulfate solution for 1-2 minutes.
10. Wash in running tap water for 10 minutes.
11. Rinse well in distilled water.
12. Counterstain with routine Harris hematoxylin and eosin stain.
13. Dehydrate in 95% alcohol, absoluta alcohol, and clear in xylene, three changes each.

14. Mount with Permount or Histoclad.

RESULTS

Basement membrana	- black
Reticulum fibers	- black
Nuclei	- blue

Cytoplasm, collagen, and connective tissue - pink to orange

REMARKS. Argentaffin cell granules stain yellow if Brentamine Fast Red B solution has broken down.

REFERENCES. Clayden, E. C.: *Practical Section Cutting and Staining,* 3rd ed., London, *W.2,* J. and A. Churchill, Ltd., 1955, p. 53.

DEFANOS METHOD FOR GOLGI APPARATUS

FIXATION. Cobalt nitrate. Use three specimens, not to exceed 1 mm in thickness.
TECHNIQUE. Cut paraffin sections at *2 -4* microns.

SOLUTIONS

FIXATIVE SOLUTION

Cobalt nitrate ..... 1.0 gm
Distilled water ..... 100.0 ml
Formalin, 37 — 40%..... 15.0 ml

1.5% SILVER NITRATE SOLUTION

Silver nitrate ..... 1.5 gm
Distilled water ..... 100.0 ml

RAMON Y CAJAL'S REDUCING SOLUTION

Hydroquinone ..... 1.0 gm
Formalin, neutral ..... 7.5 ml
Distilled water ..... 50.0 ml
Sodium sulfite ..... 0.25 gm

Make fresh.

STAINING PROCEDURE

1. Place the three specimens **in** fixative solution for *2 -4* hours.
2. Rinse in distilled water.
3. Silver nitrate solution, in the dark, for *16-24* hours. Use 300 ml of silver nitrate solution to cover all three specimens.
4. Rinse well in several changes of distilled water.
5. Place the three impregnated specimens in reducing solution and remove the first after 8 hours, the second after 16 hours, and the third after *24* hours.
6. Dehydrate specimen in graded alcohols, clear in xylene or chloroform, then infiltrate with paraffin.
7. Embed in paraffin.
S. Cut paraffin sections at *2 - 4* microns.
9. Deparaffinize in xylene, several changes.
10. Mount with Permount or Histoclad.

SMITH'S METHOD FOR JUXTAGLOMERULAR GRANULES

FIXATION. Smith's fixative.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SMITH'S FIXATIVE SOLUTION

Formalin, 10%	100.0 ml
Potassium dichromate	5.0 gm
Glacial acetic acid	2.5 ml

Follow fixation by washing in tap water for 8 hours. This fixative is not stable. It should be made fresh just before use.

1% POTASSIUM PERMANGANATE SOLUTION (STOCK)

Potassium permanganate	1.0 gm
Distilled water	100.0 ml

1% SULFURIC ACID SOLUTION (STOCK)

Sulfuric acid, concentrated	1.0 ml
Distilled water	100.0 ml

POTASSIUM PERMANGANATE-SULFURIC ACID SOLUTION (WORKING)

Potassium permanganate (stock)	50.0 MI
Sulfuric acid (stock)	50.0 ml

Mix just before use.

1% SODIUM THIOSULFATE (F~0) SOLUTION

Sodium thiosulfate	1.0 gm
Distilled water	100.0 ml

ZIEHL'S CARBOL-FUCHSIN SOLUTION

Basic fuchsin	1.0 gm
Phenol crystals	5.0 gm
Alcohol, 95%	10.0 ml
Distilled water	100.0 ml

In a mortar grind basic fuchsin and phenol crystals. Then add 95% alcohol while still grinding. The distilled water is added in 10 ml lots to wash out the mortar. The accumulated washings are filtered before use.

ACID ALCOHOL SOLUTION

Alcohol, 35%	100.0 ml
Hydrochloric acid, concentrated	0.5 ml

BOWIES SOLUTION

Biebrich scarlet	1.0 gm
Distilled water	250.0 ml

-0~

This is filtered into a 1 liter flask while the following (ethyl violet) solution is being mixed.

ETHYL VIOLET SOLUTION

Ethyl violet, ... 2.0 gm
Distilled water ..... 500.0 ml

The ethyl violet solution is then filtered into the biebrich scarlet while shaking it, until an abrupt color change (red to violet) signals the neutralization end point. The resulting precipitate is separated by vacuum filtering, dried and added to 100 ml of absolute alcohol.

ACETONE-XYLENE SOLUTION

Acetone ..... 50.0 ml
Xylene ..... 50.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in freshly prepared potassium permanganate and sulfuric acid solution for 1 minute.
3. Rinse briefly in distilled water.
4. Decolorize in sodium thiosulfate solution for 3 minutes.
5. Wash in tap water for 2 minutes.
6. Ziehl's carbol-fuchsin solution for 1 minute.
7. Wash in tap water for 1 minute.
8. Decolorize in acid alcohol solution until sections are pink. Retention of red color in some nuclei is more desirable than complete extraction.
9. Wash in tap water for 2 minutes.
10. Handle each slide individually in this and subsequent steps. Place the slide flat on a staining rack without draining or shaking off surface water. Drop on sufficient Bowie solution to cover the section without flooding the slide. After 5 to 10 seconds, immerse the slide in a coplin jar of water without draining off the dye. Wait 1 to 2 minutes before dehydrating.
11. Dehydrate rapidly through acetone for two changes and equal parts acetonexylene for one change.
12. Clear in xylene, two changes.
13. Mount with Permount or Histoclad.

RESULTS

Juxtaglomerular granules	-deep purple
Nuclei	-some pink to red, majority lavender
Cytoplasm	-shades of blue or purple
Elastic tissue	-deep purple
Collagen	-pink
Erythrocytes	-red

Note: The author (C.L.S.) has found the greatest variability in the staining results of

prepare the solution is to weigh the filter paper prior to filtering the biebrich scarlet-ethyl violet reaction mixture, then filter, dry and weigh the combination. Subtract the original paper weight to determine the amount of dye present. Place the paper and dye in a sufficient quantity of absolute alcohol to yield a 1% solution. There may still be some undissolved dye present, but this does not matter.

The mouse juxtaglomerular granules are the most prominent and easiest to stain. If good results cannot be obtained, try running the unsatisfactorily stained slide back to water and apply a second application of Bowie's solution. This will sometimes result in an improvement, if not try a different dye lot or manufacturer. It is also possible to bleach out all the stain by repeating the procedure froco Step 2. This sometimes yields better results and conserves research material.

REFERENCE. Smith, C. *L.: Stain Techn.* 41:291-294, 1966. Copyright by Williams and Wilkins Co.

FONTANA-MASSON SILVER METHOD

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SILVER NITRATE SOLUTION (FONTANA)

Dissolve 10 gm of silver nitrate in 100 ml of distilled water. To 95 ml of this solution, add ammonium hydroxide until a clear solution with no precipitate is obtained. AM, drop by drop, enough of the remaining 5 ml of silver nitrate solution to cause the clear solution above, to become slightly cloudy. Let stand overnight before using. When ready to use, dilute each 25 ml of silver solution with 75 ml of distilled water and filter.

GOLD CHLORIDE SOLUTION

Gold chloride, 1 1% aqueous	10.0 ml
Distilled water	40.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Silver nitrate solution at 56 °C for 1 hour. Sections should be light brown.
3. Rinse sections in distilled water.
4. Gold chloride solution for 10 minutes.
5. Rinse in distilled water, three changes.
6. Sodium thiosulfate solution for 5 minutes.

8. Nuclear fast red solution for 5 minutes.
9. Rinse in distilled water, two changes.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Silver reducing substances	- black
Nuclei	- pink

REMARKS. In our experiente we find that this procedure is nonspecific lince many pigments can be demonstrated, including formalin pigment. It is being used widely as an aid for the demonstration of melanin but positive results should not be interpreted as melanin pigment, unless other procedures confirm the results. Argentaffin granules are demonstrated with this method.

REFERENCE. Masson, P.: *Amer. J. Path.* 4:181-212, 1928.

MAILDONADOS METHOD FOR PANCREATIC ISLET CELLS

FIXATION. Bouin's without acetic acid.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% PHLOXINE SOLUTION

(See page 36)

3% PHOSPHOTUNGSTIC ACID SOLUTION

Phosphotungstic acid	3.0 gm
Distilled water	100.0 ml

0.05% AZURE II SOLUTION

Azure II	0.05 gm
Distilled water	100.0 ml

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Phloxine solution for 10 minutes.
3. Rinse in distilled water.
4. Phosphotungstic acid solution for 1 minute.
5. Rinse in distilled water.
6. Azure II solution for 30 seconds.
7. Rinse in distilled water.
8. Weigert's hematoxylin solution for 1 minute.

do

10. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes each.

11. Mount with Permount or Histoclad.

RESULTS

A cells - purple
B cells - violet blue
D cells - light blue with evident granules
Exocrine cells -grayish blue with red granules

REFERENCE. Maldonado, R., and San Jose, H.: *Stain Techn. 42:11 -13, 1967.* *Copy*-right by Williams and Wilkins Co.

GOMORI'S METHOD FOR PANCREATIC ISLET CELLS

FIXATION. Bouin's or 10% buffered neutral formalin. If formalin-fixed, the paraffin sections should be mordanted in Bouin's solution for *16* hours before staining. TECHNIQUE. Cut paraffin sections at *6* microns.

SOLUTIONS

BOUIN'S SOLUTION

(See page *5)*

POTASSIUM PERMANGANATE SOLUTION

Potassium permanganate ..... *0.3* gm
Distilled water ..... 100.0 ml
Sulfuric acid concentrated ..... *0.3* ml

5% SODIUM BISULFITE SOLUTION

Sodium bisulfite ..... . *5.0* gm
Distilled water ..... 100.0 ml

CHROMIUM HEMATOXYLIN SOLUTION

Hematoxylin, 1% aqueous solution ..... 50.0 ml
Chromium potassium sulfate, *3%* aqueous solution ..... 50.0 ml

To 100 ml of chromium hematoxylin solution add 0.1 gm of potassium iodate. Boil until a deep blue. The mixture is ripe immediately and can be used as long as a film with a metallic luster forros on its surface in a coplin jar. Filter before use.

1% ACID ALCOHOL SOLUTION

(See page *38)*

0.5% **PHLOXINE B** SOLUTION

Phloxine B.....0.5 gm
Distilled water ..... 100.0 ml

5% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 86)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin's solution for 16 hours.
3. Wash in tap water to remove picric acid, for 15 minutes.
4. Potassium permanganate solution for 1 minute.
5. Differentiate in sodium bisulfite solution.
6. Wash well in tap water.
7. Chromium hematoxylin solution for 10 minutes or less. Check under microscope and stain until beta cells stand out deep blue.
8. Differentiate in acid alcohol solution for 1 minute.
9. Wash in tap water until the section is a clear blue.
10. Phloxine B solution for 5 minutes.
11. Rinse in distilled water.
12. Phosphotungstic acid solution for 1 minute.
13. Wash in tap water for 5 minutes. The section should regain its red color.
14. Differentiate in 95% alcohol. If the section is too red and the alpha cells do not stand out clearly enough, rinse in 80% alcohol for 15 to 20 seconds.
15. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
16. Mount with Permount or Histoclad.

RESULTS

Alpha cells	- red
Beta cells	- blue
D cells	-from pink to red and indistinguishable froco the alpha cells.

REFERENCE. Gomori, G.: *Amer. J. Path.* 17:395 -406, 1941.

GOMORI'S METHOD FOR CHROMAFFIN

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

AZOCARMINE G SOLUTION

Azocarmine G *	0.05 gm
Glacial acetic acid	1.0 ml
Distilled water r	100.0 ml

1% ANILINE-ALCOHOL SOLUTION

Aniline	1.0 ml

3% PHOSPHOTUNGSTIC ACID SOLUTION

(Soe page, 105)

ANILINE BLUE-QUINOLINE YELLOW SOLUTION

Aniline blue	0 5 gm
Quinoline yellow or orange G	2.0 gm
Phosphotungstic acid	10 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Azocarmine G solution at 58 °C, for 60 to 90 minutes.
3. Wash in tap water, then blot.
4. Rinse in 95% alcohol.
5. Differentiate in aniline-alcohol. Check microscopically to the point where chromaffin cells stand out deep pink against the much palor cordeai cells.

Note. Differentiation in aniline alcohol solution may take 5 to 60 minutes, depending on the type of fixation and on the thickness of the section. This is the only criticai area in the entire procedure. Judging the correct degree of decolorization requires some experiente.

6. Wash briefly in tap water.
7. Phosphotungstic acid solution for 20 minutes.
8. Wash in water for 1 minute.
9. Aniline blue-quinoline yellow solution until connective tissue is deep blue — froco 15 to 40 minutes. Better contrast is obtained if quinoline yellow is used in this mixture instead of orange G.)
10. Wash **in** tap water.
11. Dehydrate in 95% alcohol, absoluto alcohol, and olear in, xylene, two changes each.
12. Mount with Permount or Histoclad.

RESULTS

chromaffin granules - purplish red

Only alpha cells of pancreatic islets, some cells of anterior pituitary and granulation of neutroplúls and myelocytes stain similarly.

REFERENCE. Gomori, G.: *Amer. J. Clin. Path.* 16:115, 1946. Copyright by Williams and Wilkins Co.

GLENNER-LILLIE METHOD FOR PITUITARY

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

STAINING SOLUTION

Eosin B, 1 % aqueous	8 0 ml
Aniline blue, 1 1% aqueous	2.0 ml

Citric acid, *0.1M* 1 1 ml
Disodium phosphate, *0.2M* 0 9 m1
Distilled water ..... *28.0* ml
Store at room temperature.

ACETONE-XYLENE SOLUTION

Acetona .....50.0 ml
Xylene.....50.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Staining solution in oven at 60 °C for 1 hour.
3. Wash in water for 5 minutes.
4. Dehydrate through 50% acetone, *80%* acetone, and 100% acetone, two changes each.
5. Clear in acetone-xylene solution, then xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Beta cell granules - blue to black
Acidophil cells - dark red
Chromophobe granules - slate gray to pale pink
Colloid - red to blue violet
Erythrocytes - orange

REMARKS. This technique is also useful for duodenal enterochromaffin cells and pancreatic islet cells with the following modification:

At step *2:* The staining solution is buffered to pH 3.6 *(O.IM* citric acid, 1.4 ml; *0.2M* disodium phosphate, 0.6 ml) and heated to 60 °C. Sections are then incubated at this temperature for 3 minutes and carried through the rest of the procedure as described above.

RESULTS

Enterochromaffin cells - blue-black
Goblet cells and mucins - light blue
Paneth cells - blue-black
Alpha cells of the Pancreatic islet - red
Beta cells of the Pancreatic islet - purple
Background - varying shades ranging froco blue to pale pink

Note. The stain can be extracted by exposure to two 5 minute changes of 1 ml *O.IN* NaOH in 39 ml *70%*

REFERENCE. Glenner, G. G., and Lillie, R. D.: *Stain Techn. 32:187* - 190, *1957.* *Copy*right by Williams and Wilkins Co.

HEATH'S METHOD FOR PITUITARY

FIXATION. 10% buffered neutral formalin, Zenker's, or Bouin's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PERFORMIC ACID SOLUTION

Hydrogen peroxide, 30% ..... 15.0 ml
Sulfuric acid, concentrated ..... 1.5 ml
Formic acid, 97% ..... 133.5 ml

4N SWLFURIC ACID SOLUTION

Sulfuric acid, concentrated ..... 112.6 ml
Distilled water ..... 887.4 ml

ALCIAN BLUE SOLUTION

Alcian blue, 4% ..... 100.0 ml
Sulfuric acid, 4N ..... 100.0 ml

0.5% PERIODIC ACID SOLUTION

(See page 72)

SCHIFF REAGENT SOLUTION

(See page 159)

0.5% ORANGE G SOLUTION

Orange G ..... 0.5 gm
Distilled water ..... 100.0 ml

1% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 74)

1 % GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid 1.0 M1
Distilled water ..... 99.0 ml
Adjust pH to 4.0 - 5.0.

STAINING PROCEDURE

1. Deparaffinize and hydrate to 70% alcohol.
2. If Zenker fixed, remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Performic acid solution for 5 minutes.
4. Wash in tap water for 10 minutes.
5. Alcian blue solution for 1 hour.
6. Wash in tap water for 10 minutes.
7. Periodic acid solution for 5 minutes.
8. Rinse in distilled water.
9. Schiff's solution for 10 minutes.
10. Wash in tepid tap water for 10 minutes.

12. Phosphotungstic acid solution for *30* seconds.
13. Glacial acetic acid solution for *30* seconds.
14. Dehydrate in absoluta alcohol, then clear in xylene, two changes each.
15. Mount with Permount or Histoclad.

RESULTS

Neurosecretory material in pars neurosa	- clear blue
Basement membranas	- magenta
Erythrocytes	- yellow
Cell cytoplasms	- chromophobic or pala magenta
Colloid	- strong magenta
Basophils	- blue, purple, or red
Acidophils	- orange to yellow

REFERENCE. Heath, E. H.: *Amer. J. Vet. Res. 26:368 -373, 1965*

LUNA'S METHOD FOR ERYTHROCYTES AND EOSINOPHIL GRANULES

FIXATION. 10% buffered formalin.

TECHNIQUE. Cut paraffin sections at *6* microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTIONS

(See page *35)*

1% BIEBRICH SCARLET SOLUTION

Biebrich scarlet ..... 1.0 gm
Distilled water ..... 100.0 ml

HEMATOXYLIN-BIEBRICH SCARLET SOLUTION (WORKING)

Weigert's irou hematoxylin solution (working)..... 45.0 ml
Biebrich scarlet solution ..... 5.0 ml

1% ACID ALCOHOL SOLUTION

(See page *38)*

0.5% LITHIUM CARBONATE SOLUTION

Lithium carbonate .....0.5 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Stain in working hematoxylin-biebrich scarlet solution for *5* minutes.
3. Differentiate in 1% acid alcohol until desired nuclear detail is achieved (usually eight dips).
4. Rinse in tap water to remove acid alcohol.

5. Dip lithium carbonate solution until section turns blue and erythrocytes are bright red (usually five dips).

6. Wash in running water for 2 minutes.

7. Dehydrate in 95% alcohol, absoluto alcohol, and clear in xylene, two changes each.

8. Mount with Permount or Histoclad.

RESULTS

Eosinophil granules	- red
Erythrocytes	-red
Charcott Leyden crystals	-red
Background	- blue

REMARKS. Especially useful or phagocytosis and sometimes useful for demonstrating Negri bodies.

REFERENCE. Luna, L. G.: Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C. 20305.

MONROE-FROMMER METHOD FOR PITUITARY

FIXATION. Zenker's

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

10% TANNIC ACID SOLUTION

Tannic acid	10.0 gm
Distilled water	100.0 ml

1 % BASIC FUCHSIN SOLUTION (STOCK)

Basic fuchsin	10 gm
Alcohol, 100%	20.0 ml
Distilled water	80.0 ml

BASIC FUCHSIN SOLUTION (WORKING)

Basic fuchsin (stock)	50.0 ml
Distilled water	50.0 ml

Filter before use.

1% ANILINE SOLUTION

Aniline	1.0 ml
Alcohol, 100%	90.0 MI
Distilled water	10.0 ml

1% PHOSPHOMOLYBDIC ACID SOLUTION

Phosphomolybdic acid	10 gm
Distilled water	100.0 ml

1% ALCIAN **BLUE** SOLUTION

Alcian blue, 8GX	10 gm
Distilled water	100.0 Ml.

Filter before use.

STAINING PROCEDURE

Deparaffinize and hydrate to distilled water. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).

Wash in running water for 10 minutes.

Tannic acid solution for 10 minutes.

Wash in running water for 5 minutes.

Working basic fuchsin solution for 3-5 seconds Slides should be agitated by dipping in and out of staining solution.

7. Wash in tap water to remove excess stain.
8. Differentiate in aniline solution until acidophils are red and basophils pink.
9. Phosphomolybdic acid solution for 30 seconds.
10. Rinse in distilled water.
11. Alcian blue solution for 30 seconds.
12. Rinse in distilled water.
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Acidophils	– red
Beta basophils	–green
Delta basophils	- purple
Collagenous fibers	- bluish green
Chromophobes	- colorless
Colloid	-red
Neurosecretory granules	- brilhant red
Neurohypophysial fibers	- blue

REFERENCE. Monroe, C. W. and Frommer, J.: *Stain Techn.* 41:248, 1966. Copyright by Williams and Wilkins Co.

WILSON-EZRIN METHOD FOR PITUITARY

FIXATION. 10% formol-salive solution.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% PERIODIC ACID SOLUTION

Periodic acid	10 gm
Distilled water	100.0 ml

SCHIFF REAGENT SOLUTION

(See page 159)

SULFUROUS ACID SOLUTION

Sodium metabisulfite, 10% aqueous ..... 60.0 ml
Hydrochloric acid, IN ..... 50. 0 ml
Distilled water ..... 1000.0 ml

1 % ORANGE G SOLUTION

Orange G ..... 1.0 gm
Distilled water ..... 100.0 ml

5% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 86)

1% METHYL BLUE SOLUTION

Methyl blue ..... 1.0 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1.. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 5 minutes.
3. Rinse in distilled water.
4. Schiff's solution for 15 minutes.
5. Sulfurous acid rinses, three changes, 3 minutes each.
6. Wash in running water for 10 minutes.
7. Orange G solution for 1 minute. S.

Phosphotungstic acid solution for 30 seconds.

9. Wash in running water for 30 seconds.
10. Methyl blue solution for 1 minute.
11. Wash out excess methyl blue in tap water.
12. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Beta granules - red
Gamma granules - purple
Acidophils - yellow

REFERENCE. Wilson, W. D., and Ezrin, E.: *Amer. J. Path.* 30:891-899, 1954.

LUNAS METHOD FOR MAST CELLS

FIXATION. 10% buffered neutral formalin

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALDEHYDE FUCHSIN SOLUTION

(See page 78)

WE1GERTS IRON HEMATOXYLIN SOLUTION

(See page 35)

METHYL ORANGE SOLUTION

Methyl orange	0.25 gm
Alcohol, 95%	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to 95% alcohol.
2. Aldehyde fuchsin solution for 30 minutes.
3. Rinse in 95% alcohol.
4. Weigert's hematoxylin solution for 1 minute.
5. Wash in running water for 10 minutes.
6. Rinse in 95% alcohol.
7. Counterstain in methyl orange solution for 5 minutes or until background is light yellow.
8. Dehydrate in absoluto alcohol, then clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Mast cells	- purple
Elastic fibers	- purple
Other cellular elements	- blue
Background	- yellow

REMARKS. Especially useful for demonstration of mast cells in animal tissue. REFERENCE. Luna, L. G.: Histopathology Laboratories, Armed Forces Instituto of Pathology Washington, D. C. 20305.

UNNA'S METHOD FOR MAST CELLS

FIXATION. 10% buffered neutral formalin or formol alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

POLYCHROME METHYLENE BLUE SOLUTION

Methylene blue	1.0 gm
Distilled water	100.0 ml
Alcohol 95%	20.0 ml
Potassium carbonate	1.0 gm

GLYCERIN-ETHER SOLUTION

Glycerin ..... 50.0 ml
Calcium chloride anhydrous ..... 10.0 gm

Heat the above inixture until the calcium chloride dissolves completely. Cool to room temperatura.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Polychrome methylene blue solution for 10 minutes.

S. Rinse in distilled water.

4. Differentiate in glycerin-ether solution, diluted 5 to 10 times with distilled water, for 30 seconds to 1 minute, or until, section is a medium blue. Be careful not to differentiate too long.
5. Wash thoroughly in water for 2 -5 minutes, then blot with filter papar.
6. Dehydrate rapidly in absoluta alcohol.
7. Clear in xylene, two or three changes.

S. Mount with Permount or Histoclad.

RESULTS

Mast cell granules -red
Other cells - greenish blue

REMARKS. This technic, which was originally used for the differentiation of mast cells and plasma cells, has been used in our laboratory for the specific demonstration of mast cells. It is especially useful for highly cellular specimens, such as lymph node and spleen.

REFERENCE. Mallory, F. B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 175.

TORENS METHOD FOR MAST CELLS

FIXATION. 10% buffered neutral. formalin.

TECHNIQUE. Cut paraffin sections at 6 microns-

ìi SOLUTIONS

GIEMSA SOLUTION

Hartman-Leddon Company original ature blend type Giemsa staining solution 1 drop per nil of distilled water, prepared immediately bafore use.

0.5% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid ..... 0.5 ml
Distilled water ..... 100.0 ml

POLLACK'S TRICHROME SOLUTION

Acid fuchsin ..... 0.5 gm
Ponceau 2 R ..... 1.0 gm

Light green, SF yellowish	0.45 gm
Orange G	0.75 gm
Phosphotungstic acid,C. P.	1.5 gm
Phosphomolybdic acid,C. P.	1.5 gm
Glacial acetic acid	3.0 ml
Alcohol, 50% to make	—
	300.0 ml

First, glacial acetic acid is added to the alcohol. Of this acidified alcohol, 50 ml portions are prepared in four beakers: in the first beaker, acid fuchsin and ponceau 2R are dissolved; in the second, light green; in the third, orange G and phosphotungstic acid; and in the fourth, phosphomolybdic acid. The rest of the acidified alcohol is used to rinse out the beakers and to make up the volume. All the ingrediente dissolve easily except for the phosphomolybdic acid, which will dissolve after slight warming of the beaker.

The mixture is filtered and then ready for use.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Giemsa solution for 75 minutes.
3. Rinse in distilled water.
4. Glacial acetic acid solution for 5 to 10 seconds.
5. Rinse in distilled water.
6. Pollack's trichrome solution for 10 to 15 seconds.
7. Rinse in glacial acetic acid solution until no more stain washes off, 3 to 5 seconds.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad

RESULTS

Mast cell granules	- deep blue
Collagen and cartilage	- blue-green
Muscle and elastic fibers	-red
Fibrin	- purple
Bone	- green
Colloid, keratin and erythrocytes	- orange red to orange

REFERENCE. Toren, D. A.: *Stain Techn.* 38:249 -250, 1963. Copyright by Williams and Wilkins Co.

BERG'S METHOD FOR SPERMATOZOA

FIXATION. 10% buffered neutral formalin.

TECH N 1 QU E. Cut paraffin sections at 6 microns.

SOLUTIONS

PUTT'S CARBOL, FUCHSIN SOLUTION

New fuchsin	10 gm
Phenol	5 0 gm

Alcohol, 100% ..... 10.0 ml
Distilled water ..... 84.0 ml

Filter before use.

SATURATED LITHIUM CARBONATE SOLUTION

(See page 38)

5% GLACIAL ACETIC ACID-ALCOHOL SOLUTION

Glacial acetic acid ..... 5.0 ml
Alcohol, 100% ..... 95.0 ml

0.5% METHYLENE BLUE SOLUTION

Methylene blue ..... 0.5 gm
Alcohol, 100% ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Carbol fuchsin solution for 3 minutes.
3. Place slides directly finto saturated lithium carbonate solution for 3 minutes.
4. Decolorize in glacial acetic acid-alcohol solution for 5 minutes.
5. Absolute alcohol, two changes for 1 minute each.
6. Methylene blue for 30 - 60 seconds.
7. Rinse rapidly in absolute alcohol, two changes.
8. Clear in xylene, two or three changes.
9. Mount with Permount or Histoclad.

Note. Uneven staining can be avoided if slides are gently agitated throughout all stages of the procedure.

RESULTS

Spermatozoa - brilliant red
Erythrocytes - pale pink
Other tissue componente - blue to purple

REFERENCE. Berg, J. W.: *Amer. J. Clin. Path.* 23:513-515, 1963. Copyright by Wil-

Chapter 8

Methods For Hematologic And Nuclear Elements

WOLBACH'S GIEMSA METHOD

FIXATION. Zenker's or other we11-fixed tissues.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

GIEMSA* SOLUTION (STOCK)

Giemsa powder	1.0 gm
Glycerin	66.0 ml
Alcohol, methyl	66.0 ml

Mix glycerin and Giemsa powder. Place in a 60 °C oven for 2 hours. Finally add the 66 ml methyl alcohol.

GIEMSA SOLUTION (WORKING)

Giemsa solution (stock)	1.25 ml
Alcohol, methyl	1.5 ml
Distilled water	50.0 ml

ROSIN ALCOHOL SOLUTION (STOCK)

Rosin, white	10.0 gm
Alcohol, 100%	100.0 ml

ROSIN ALCOHOL SOLUTION (WORKING)

Rosin solution (stock)	5.0 ml
Alcohol, 95%	40.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Wash in running water for 15 minutes.
4. Rinse in distilled water.
5. Working Giemsa solution overnight.
6. Differentiate in working rosin alcohol solution until sections assume a purplish pink color. Check under microscope.

OStain must be National Aniline Certified Giemsa.

7. Dehydrate in absoluto alcohol then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Nuclei	- blue
Collagen and other tissue elements	- pink to rose
Rickettsia	- purple
Bacteria	- blue

REMARKS. Rickettsia can be demonstrated satisfactorily with this method. The demonstration, however, depends on precise differentiation of the section.

Note. Giemsa takes much better if tissue has an acid pH. If tissue is not already acidified by decalcification, etc., placo in acid alcohol, wash well, begin stain.

REFERENCE. Wolbach, S. B., Todd, J. L., and Palfrey, *F. W.: The Etiology of Pathology Typhus,* Cambridge, Mass., Harvard University Press, *1922, pp. 13 -14.*

MAXIMOW'S METHOD FOR BONÉ MARROW

FIXATION. Zenker-Formol.

TECHNIQUE. Cut parrafin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(Soe pago *33)*

0.1% EOSIN SOLUTION (STOCK)

Eosin Y, yellowish	0.1 gm
Distilled water	100.0 ml

0.1% AZURE **11** SOLUTION (STOCK)

Azure II	0.1 gm
Distilled water	100.0 ml

EOSIN-AZURE II SOLUTION (WORKING)

Eosin solution (stock)	10.0 ml
Distilled water, triple distilled	100.0 ml
Azure II solution (stock)	10.0 ml
Glacial acetic acid	2 drops

Must be prepared fresh each time with triple distilled water or buffer solution pH 6.8 - 7.0.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 15 minutes. S. Wash in tepid tap water for 15 minutes. 4. Working Eosin-Azure II solution overnight.

5. Differentiate in 95% alcohol until blue ceases to come out finto alcohol and erythrocytes and collagen are pink.
6. Dehydrate in absolute alcohol, clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Nuclei	- blue
Basophile leucocyte, mast cell granules	-purple to violet
Cartilage	purple
Erythrocytes, eosinophile granules	pink
Cytoplasm	blue to pink

REFERENCE. Mallory, F. B.: Pathological. Technique, New York, Hafner Publishing Co., 1961, p. 196.

MAY - GRUNWALD GIEMSA METHOD

FIXATION. Zenker's or other well-fixed tissue.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

JENNER SOLUTION (STOCK)

Jenner stain, dry powder"	1.0 gm
Alcohol, methyl	400.0 ml

JENNER SOLUTION (WORKING)

Jenner solution (stock)	25.0 ml
Distilled water	25.0 ml

GIEMSA SOLUTION (STOCK)

(See page 119)

GIEMSA SOLUTION (WORKING)

Giemsa solution (stock)	50 drops
Distilled water	50.0 ml

Make fresh, do not re- use.

1% GLACIAL ACETIC WATER SOLUTION

(See page 94)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thio-

3. Wash in running water for 10 minutes.
4. Rinse in distilled water, two changes.
5. Methyl alcohol, two changes for 3 minutes each.
6. Working Jenner solution for 6 minutes.
7. Working Giemsa solution for 45 minutes.
8. Handle each slide individually in this and subsequent steps. Differentiate in glacial acetic water solution then check microscopically for well differentiated nuclei.
9. Rinse in distilled water.
10. Dehydrate quickly in 95% alcohol, absolute alcohol, and clear with xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Nuclei - blue
Cytoplasm - pink to rose
Bactéria - blue

REFERENCE. Strumia, M. M.: *J. Lab. Clin. Med.* 21:930 - 934, 1935 -1936.

MALLORY'S METHOD FOR HEMOFUCHSIN

FIXATION. Zenker's solution, absolute alcohol or 10% buffered neutral formalin. TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALUM HEMATOXYLIN SOLUTION

Hematoxylin	1.0 gm
Alummum ammonium or potassium sulfate	20.0 gm
Distilled water .	400.0 ml
Thymol	1.0 gm

0.5% BASIC FUCHSIN SOULTION

Basic fuchsin	0.5 gm
Alcohol, 95%	50.0 ml
Distilled water	50.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Alum hematoxylin solution until the nuclei stand out sharply.
3. Wash thoroughly in water.
4, Basic fuchsin solution for 30 minutes.
5. Wash in water.
6. Differentiate in 95% alcohol until hemofuchsin granules stand out sharply against a gray background.
7. Dehydrate in absolute alcohol, then clear in xylene, two changes each.

RESULTS

Nuclei - blue
Hemofuchsin granules - bright red

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 136.

LISON'S METHOD FOR HEMOGLOBIN

FIXATION. 10% buffered neutral. formalin
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

Patent Blue B PATENT BLUE B SOLUTION (STOCK)..... 1.0 gm

Glacial acetic acid, 2% ..... 100.0 ml
Zinc dust, granulated ..... 10.0 gm
Boil until pale brown. Filter.

PATENT BLUE B SOLUTION (WORKING)

Patent Blue B solution ..... 100.0 ml
Glacial acetic acid ..... 20.0 ml
Hydrogen peroxide, 3% ..... 10.0 ml

SAFRANIN O SOLUTION

Safranin O 0 1 gm
Glacial acetic acid, 1% ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to 70% alcohol.
2. Working Patent blue B solution for 3 minutes.
3. Rinse in distilled water.
4. Safranin O solution for 1 minute.
5. Rinse in distilled water and blot dry.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Hemoglobin -dark blue to green
Granules of leucocytes -dark blue
Nuclei and cytoplasm - red

Note: This technic may be used for coloring of myeloid white blood cell peroxidase granules.

REFERENCE. McManus, J. F. A., and Mowry, R. *W.: Staining Methods Histologic and Histochemical,* New York, Hoeber Medicai Division, Harper and Row, Publishers, 1960, p. 204.

PUCHTLER METHOD FOR HEMOGLOBIN

FIXATION. Zenker's solution.

TECHNIQUE. Cut parrafin sections at 6 microns.

SOLUTIONS

5% TANNIC ACID SOLUTION

Tannic acid	5.0 gm
Distilled water	100.0 ml

Tannic acid prepared fresh should be allowed to stand for 48 hours before use. (stable).

1% PHOSPHOMOLYBDIC ACID SOLUTION

(See page 112)

BUFFALO BLACK NBR 126% SOLUTION

Buffalo Black NBR, concentrated *126%**	3.15 gm
Glacial acetic acid	10.0 ml
Alcohol, methyl	90.0 ml

Solution must be allowed to stand for 48 hours before use. DO NOT FILTER BEFORE USE.

METHANOL -GLACIAL ACETIC ACID SOLUTION

(See page 126)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Zenker-formol overnight.
3. Wash in running water for *15* minutes.
4. Remove mercuric chloride crystals with iodine and clear with sodium thiosulf ate (see page 41).
5. Wash in running water for 5 minutes.
6. Tannic acid solution for 10 minutes.
7. Rinse in distilled water, three changes.
8. Phosphomolybdic acid solution for 10 minutes.
9. Rinse in methanol-glacial acetic acid, two changes.
10. Buffalo black NBR solution for *5* minutes. *DO NOT FILTER.*
11. Rinse in methanol-glacial acetic acid, two changes.
12. Dehydrate in absolute alcohol, then clear in xylene, two or three changes each.
13. Mount with Permount or Histoclad.

*National Aniline Division, 201 W. First St., Charlotte, N. C. 28801

RESULTS

Erythrocytes	-dark blue
Hemoglobin casts	- dark blue
Intracellular hemoglobin droplets	- dark blue
Other tissue structures	-yellow

REMARKS. In our experiente we find this technic to be the best available for the demonstration of hemoglobin — only if tissue is Zenker fixed.

Note. Buffalo Black NBR concentrated 126% must be used to insure satisfactory results.

REFERENCE. Puchtler, H., Rosenthal, S., and Sweat, *F.: Arch. Path. 78:76-78, 1964.*

RALPH'S METHOD FOR HEMOGLOBIN

FIXATION. Absolute alcohol, Carnoy's solution or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at *6* microns.

SOLUTIONS

BENZIDINE SOLUTION

Benzidine	1.0 gm
Alcohol, methyl	*99.0* ml

PEROXIDE SOLUTION

Hydrogen peroxide, 30/07,,	25.0 ml
Alcohol, *70%*	75.0

ml Handle hydrogen peroxide with Gare, avoid contact with skin and eyes.

NUCLEAR FAST **RED (KERNECHTROT) SOLUTION**

(See page *89)*

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Flood slide with benzidine solution for 1 minute.
3. Drain off and flood with the peroxide solution for 2 minutes.
4. Rinse in distilled water for *15* seconds.
5. Nuclear fast red for *5* mintues.
6. Rinse in distilled water.
7. Dehydrate in 95% alcohol, absolute alcohol, and olear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Hemoglobin	- dark brown
Nuclei	- pink

REFERENCE. Ralph, P. H.: *Stain Techn. 16:105, 1941.* Copyright by Williams and Wilkins Co.

PUCHTLER-SWEAT METHOD FOR HEMOGLOBIN AND HEMOSIDERIN

FIXATION. Zenker-Formol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

2% POTASSIUM FERROCYANIDE SOLUTION (STOCK)

Potassium ferrocyanide	2.0 gm
Distilled water	100.0 ml

2% HYDROCHLORIC ACID SOLUTION (STOCK)

Hydrochloric acid, concentrated	2.0 ml
Distilled water	100.0 ml

POTASSIUM FERROCYANIDE-HYDROCHLORIC ACID SOLUTION (WORKING)

Potassium ferrocyanide (stock)	50.0 ml
Hydrochloric acid (stock)	50.0 ml

Mix just before use.

5% TANNIC ACID SOLUTION

(See page 124)

1% PHOSPHOMOLYBDIC ACID SOLUTION

(See page 112)

PHLOXINE B SOLUTION

Phloxine B	5.0 gm
Glacial acetic acid	30.0 ml
Alcohol, methyl	80.0 ml

Adjust pH to 3.7.

METHANOL- GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	10.0 ml
Alcohol, methyl	*90.0* ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Zenker-formol, overnight.
3. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
4. Rinse in distilled water, several changes. Avoid any contact with metal.
5. Potassium ferrocyanide-hydrochloric acid solution for 30 minutes.
6. Rinse in distilled water, three changes.

Ar-

7. Tannic acid solution for 10 minutes.
8. Rinse in distilled water, three changes.
9. Phosphomolybdic acid solution for 10 minutes.
10. Rinse in distilled water, three changes.
11. Phloxine B solution for 5 minutes. *DO NOT FILTER.*
12. Rinse in methanol-glacial acetic acid solution, two changes.
13. Dehydrate in absolute alcohol and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Hemoglobin - red

Hemosiderin - dark blue to blue green

REFERENCE. Puchtler, H. and Sweat, F.: *Arch. Path.* 75: 588 -590, 1963.

PRICES GIEMSA METHOD

FIXATION. 10% buffered neutral formalin or Zenker's Solution. Avoid fixatives which destroy erythrocytes.

TECHNIQUE. Cut paraffin sections at 4 microns.

SOLUTIONS

GIEMSA SOLUTION (STOCK)

	VOLUME	WEIGHT
Giemsa Powder[1]	1.0 gm	1.0 gm
Methanol	65.0 ml	51.0 gm
Glycerin	40.0 ml	51.0 gm

Measure volume of methanol. Weigh volume of methanol. Weight an equal amount of glycerin. Pour glycerin into an acid clean brown bottle. Use the measured methanol to rinse the glycerin, froin container and into the bottle. Add sterile glass beads to glycerin and methanol mixture. Combine the methanol and glycerin by shaking slightly. Add giemsa powder, tighten cap, and *shake well.* Label (Record lot no. on label). SHAKE WELL about once an hour every day for THREE DAYS! It is best to make a large volume (100 ml) of this stock and store in this bottle. When ready to use, filter through coarse filter paper into small 50 ml dark dropper bottles for use as working solutions.

GIEMSA STAIN

Label:

Giemsa: 5g. Lot No. _____ Cat. No _____
Allied Chemical, National. Aniline Div.

Glycerin: 200 ml (255 g.)
Cat. No. Lot No _____
Fisher (A.C.S.)

Methanol: 325 ml (255 g.)
Cat. No. Lot No _____
Fisher (A.C.S.)

Date _____ Made by: _____

BUFFER SOLUTION

Disodium monohydrogen phosphate anhydrous, Na_2HPO_4 ... 6.0 gm
Monopotassium di-hydrogen phosphate, KH_2PO_4 ..... 5.0 gin

Mix buffer salts and weigh out 1 gram units and placo them in small well stoppered vials or test tubos. One of these units is dissolved in one liter of distilled water to give a buffer solution of approximately pH 7.0.

GIEMSA SOLUTION (WORKING)

Giemsa (Stock) ..... 3.0 ml
Buffer solution ..... 97.0 ml

To avoid contamination of the stock solution *never* introduce a pipette into the stock container. If dropper bottles are not used, pour a small amount of solution from the stock bottle into another container and pipette from this second container the required amount. Discard the unused portion of Giemsa stock solution.

GLACIAL ACETIC ACID WATER SOLUTION

Glacial acetic acid ..... 1.0 ml
Distilled water ..... 499.0 ml

STAINING PROCEDURE

1. Deparaffinize through xylene for 2 changes, 2 minutes each.
2. Absolute alcohol for 2 changes, 2 minutes each.
3. 95% alcohol for 1 minute.
4. 95% alcohol, 2 changes.

If necessary, remove mercuric chloride crystals with alcoholic iodine. (Iodine is removed with subsequent alcohols).

5. 80%, 70%, 50% alcohol for 1 change, 1 minute each.
6. Rinse in distilled water for 15 seconds.
7. Buffer solution for 30 minutes.
8. Working Giemsa solution overnight.
9. Quick rinse in buffer solution.
10. Glacial acetic acid water solution for 1 minute. (Soe note).
11. Absolute alcohol for 2 changes, 15 seconds each.
12. Xylene for 2 changes, 2 minutes each.
13. Mount with Permount or Histoclad.

RESULTS

Malarial parasites	– blue
Malarial pigment	– black
Nuclei of tissue cells	– blue
Collagen and other elements	– pale pink
Bacteria	- dark blue
Egg shells of schistosomes	- blue
Erythrocytes	-pink to rose

NOTE: *Total time* for steps #10 and #11 should be exactly 90 seconds.

REFERENCE. Price, D. L: Mil. Med. 133: 363 -367, 1968.

THOMAS' METHOD FOR MALARIAL PARASITES

FIXATION. Any well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

PHLOXINE B SOLUTION

Phloxine *B*	0.5 gm
Distilled water	100.0 ml
Glacial acetic acid	0.2 ml

A slight precipitate forros but settles on the bottom. Filter before use.

METHYLENE BLUE-AZURE B SOLUTION

Methylene blue	0.25 gm
Azure B	0.25 gm
Borax	0.25 gm
Distilled water	100.0 ml

0.2% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	0.2 ml
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Phloxine B solution for *2* minutes.
3. Rinse *well* in distilled water.
4. Methylene blue-azure B solution for 1 minute.
5. Differentiate with glacial acetic acid solution.
6. Complete differentiation in *95%* alcohol, three changes.
7. Dehydrate in absolute alcohol, then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Nuclei - blue
Plasma cell cytoplasm - blue
Malarial parasites - blue
Erythrocytes - pink
Other tissue elements - shades of rose to red.

Note. This procedure is being used in our laboratories for the demonstration of malaria parasites in paraffin sections.

REFERENCE. Thomas, J. T.: *Stain Techn. 28:311-312, 1953.* Copyright by Williams and Wilkins Co.

CAIN'S METHOD FOR MITOCHONDRIA

FIXATION. Regaud's solution.

TECHNIQUE. Cut paraffin sections at 3 microns.

J

SOLUTIONS

REGAUD'S SOLUTION

Potassium dichromate, 3% .....80.0 ml
Formalin, 37 — 401v .....20.0 ml

Fix for 4 days, fresh solution each day; follow with 3% potassium dichromate for 8 days, changing solution every 2 days. Wash for 24 hours.

ANILINE-ACID FUCHSIN SOLUTION

Aniline ..... 1.0 ml
Distilled water .....20.0 ml
Acid fuchsin ..... 4.0 gm

Shake aniline with distilled water for a couple of minutes. Let stand and then shake again several times at intervals over 24 hours. Filter. Add acid fuchsin and shake at intervals over several hours. This solution keeps only long enough to be used the same day it is made.

0.1 % SODI UM CARBONATE SOLUTION

Sodium carbonate .....0.1 gm
Distilled water ..... 100.0 ml

1% HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, concentrated .....1.0 ml
Distilled water .....99.0 ml

0.5% METHYL BLUE SOLUTION

Methyl blue .....0.5 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Hot aniline-acid fuchsin solution for 5 -10 minutes. Heat aniline-acid fuchsin solution to steaming then remove solution froco heat when slides are placed in it.
3. Differentiate in sodium carbonate solution until cytoplasm is pale pink.
4. Dip briefly in hydrochloric acid solution to stop differentiation and also heighten color.
5. Rinse well in distilled water.
6. Counterstain in methyl blue solution for a few seconds.
7. Rinse in distilled water.
S. Dip briefly in hydrochloric acid solution.
9. Rinse in distilled water.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Mitochondria - bright red
Nuclei - blue to green

REFERENCE. Cain, A. J.: *Quart. J. Micr. Sci. 89:229-231,* 1948.

SIEGEL'S POLYCHROMATIC METHOD

FIXATION. 10% buffered neutral formalin. TECHNIQUE.
Cut paraffin sections at *6* microns.

SOLUTIONS

TOLUIDINE BLUE O SOLUTION

Toluidine blue O	1 gm
Phosphate buffer, *0.067M,* pH7.3 - 7.4	100.0 ml

NAPHTHOL YELLOW S SOLUTION

Naphthol yellow S	1.0 gm
Glacial acetic acid, 11%	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Toluidine blue O solution for 1 minute.
3. Wash in tap water.
4. Check microscopically for metachromasia.
5. Naphthol yellow S solution, one dip.
6. Wash in tap water.
7. Repeat steps 4 and 5 until brown colors are visible.
8. Dehydrate in tertiary butyl alcohol and clear in xylene, three changes, *2* minutes each.
9. Mount with Permount or Histoclad.

RESULTS

A striking spectrum of colors aid in the differentiation of various cells and tissue constituents.

Note. The optimum time of immersion in each dye solution should be determined empirically. Overstaining with toluidine blue O causes an excessive accumulation of dye in the blue or brown areas, while overstaining with naphthol yellow S can completely remove the basic stain froco some structure.

REFERENCE. Siegel, I.: *Stain Techn. 42:29 -30, 1967.* Copyright by Williams and Wilkins Co.

LILLIE'S METHOD FOR NUCLEIC ACIDS

FIXATION. 10% buffered neutral formalin. TECHNIQUE.
Cut paraffin sections at *6* microns.

SOLUTIONS

SCHIFF REAGENT SOLUTION

(see page *159)*

NORMAL HYDROCHLORIC ACID SOLUTION

(See page 158)

0.05M SODIUM BISULFITE SOLUTION

Sodium bisulfite	0.52 gm
Distilled water	100.0 ml

0.01% FAST GREEN SOLUTION

Fast green, FCF	0.01 gm
Alcohol, 95%	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Place slides in preheated normal hydrochloric acid solution at 60 °C for 10 to 15 minutes.
3. Schiff reagent solution for 10 minutes.
4. Sodium bisulfite solution, three suecessive baths for 2 minutes each.
5. Wash in running water for 5 minutes.
6. Fast green solution for a few seconds.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Nuclear chomatin	- deep red purple
The chromatin of plasmodia, sarcosporidia, toxoplasmata and histoplasmata	- pale red
Cytoplasm	- light green

Note. The fast green solution does not wash out in alcohol, but if it is too intense it may be removed promptly in water.

REFERENCE. Lillie, R. D.: *Histopathologic Technic and Practical Histochemistry,* 3rd ed., New York, McGraw-Hill Book Co., 1965, pp. 149 -150. Used by permission of McGraw-Hill, Inc.

MENZIES' METHOD FOR NUCLEIC ACIDS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

HYDROCHLORIC ACID-TETRAHYDROFURAN SOLUTION

Hydrochloric acid, concentrated	10.0 ml
Tetrahydrofuran	90.0 ml

1 % AZURE B SOLUTION (STOCK)

Azure B	1.0 gm
Distilled water	100.0 ml

0.1% BASIC FUCHSIN SOLUTION (STOCK)

Basic fuchsin .....,0.1 gm
Distilled water ..... 100.0 ml

AZURE B-BASIC FUCHSIN SOLUTION (WORKING)

Azure B (stock).....30.0 ml
Basic fuchsin (stock) ..... 8.0 ml
Glacial acetic acid ..... 2.0 ml

STAINING **PROCEDURE**

1. Deparaffinize and hydrate to distilled water.
2. Hydrochloric acid-tetrahydrofuran solution at 37 ° C for 2 - 3 minutes.
3. Working Azure B-Basic fuchsin solution for 15 -30 minutjs.
4. Rinse in acetone, clear in xylene, two to three changes each.
5. Mount with Permount or Histoclad.

RESULTS

DNA - red
RNA - blue

Note: This method is excellent for rat DNA and RNA. May be used on human tissue but the results are not as good. For human material, the following referente is recommended: Menzies, D. W.: *Stain Techn. 41:* 165 -167, 1966.

REFERENCE. Menzies, D. W.: *Stain Techn.* 38:157 -160, 1963. Copyright by Williams and Wilkins Co.

SPICER'S METHOD FOR NUCLEIC ACIDS

FIXATION. Bouin's solution.

TECHNIQUE. Cut paraffin sections at 4 microns.

SOLUTIONS

SCHIFF REAGENT SOLUTION

(See page 159)

0.5% SODIUM METABISULFITE SOLUTION

Sodium metabisulfite 0 5 gm
Distilled water ..... 100.0 ml

O.IM CITRIC ACID SOLUTION

Citric acid, anhydrous .....19.21 gm
Distilled water, to make..... 1000.0 ml

0.2M DISODIUM PHOSPHATE SOLUTION

Disodium phosphate ..... 28.40 gm
Distilled water, to make..... 1000.0 ml

METHYLENE BLUE SOLUTION

Methylene blue	0.04 gm
Citric acid, 0.2M	28.6 ml
Disodium phosphate, 0.2M	11.4 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water, wash for 5 minutes.
2. Schiff reagent solution for 10 minutes.
3. Rinse in three changes of sodium metabisulfite solution 2 minutes each.
4. Wash in running water for 10 minutes.
5. Methylene blue solution for 30 minutes. Overstaining by this solution will obscure the red in the DNA.
6. Dehydrate in 70%, 80%,100/07o acetone, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

DNA	- red
Chromosomes in mitosis	- red
Chromatin in interphase nuclei	- red
Cytoplasmic RNA	- blue

Note. For viewing sections use slightly yellowish unfiltered light from incandescant light source.

REFERENCE. Spicer, *S. S.: Stain Techn.* 36:337 -340, 1961. Copyright by Williams and Wilkins Co.

TAFT'S METHOD FOR NUCLEIC ACIDS

FIXATION. Carnoy's or absolute alcohol.

TECHNIQUE. Cut paraffin sections at 4 microns.

SOLUTIONS

METHYL GREEN - PYRONIN SOLUTION

Methyl green, purified*	0.52 gm
Pyronin Yt	0.1 gm

Heat 100 ml of distilled water to boiling in 250 ml flask. Remove from heat and add dyes. Mix thoroughly. Stopper flask and store for 5-7 days. Solution is then ready for use and should be stored in an amber glass-stoppered bottle.

DIFFERENTIATING SOLUTION

Tertiary butyl alcohol	30.0 ml
Alcohol, 100%	10.0 ml

*Methyl Green should be extracted several times with clilorofonn until extracting solution is *blue green* instead of lavender.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Methyl green-pyronin solution for 10 minutes. Filter before use.
3. Rinse briefly twice in distilled water.
4. Blot with several thicknesses of smooth filter paper.
5. Differentiating solution for 1- *2* minutes.
6. Dehydrate in a fresh change of the differentiating solution. (Slides may be left in this solution for 4 - *5* minutes without additional differentiation taking place).
7. Clear in xylene, for two changes, 10 minutes each.
8. Mount with Permount or Histoclad.

RESULTS

DNA	- blue green
RNA	- bright red
Remainder of tissue	- pale pink

REMARKS. For more information on Methyl Green-Pyronin staining, see referente books by Gomori and Pearse. Good information may also be found in the following articles:

1. Kurnick, N. B.: *Stain Techn. 30:213-230, 1955.*
2. Taft, E. B.: *Exp. Ce 11. Res. 2:312 -326, 1951.*

REFERENCE. Taft, E.B.: *Stain Techn. 26:205-212, 1951.* Copyright by Williams i and WilkinsCo.

DEOXYRIBONUCLEASE DIGESTION METHOD

FIXATION. 10% buffered neutral formalin, Carnoy's or Bouins. (Fixatives containing mercuric, chromic, or other heavy metal ions cannot be used).

TECHNIQUE. Cut paraffin sections at *6* microns.

SOLUTIONS

DEOXYRIBONUCLEASE SOLUTION

Deoxyribonuclease, cyrstalline	0.001 gm
0.2M Magnesium chloride (4.066/07o $M_9C_{12}{}'_6H_{20}$)	*22.5* ml
0.2 M Calcium chloride *(2.22%* CaCI	*2.5* ml
0.1M Tris buffer, pH *7.3*	20.0 ml
Distilled water	55.0 ml

TRIS BUFFER SOLUTION (STOCK)

Solution A

Trihydroxymethylaminomethane	*24.228* gm
Distilled water	1000.0 ml

Solution B

Hydrochloric acid, concentrated (Sp. Gr. *1.1837).*	8.35 ml
Distilled water	*991.7 ml*

—Jor

TRIS BUFFER SOLUTION pH 7.3 (WORKING)

Solution A	10.0 ml
Solution B	17.0 ml
Distilled water	13.0 ml

DIGESTION PROCEDURE. Run two control sections.

1. Deparaffinize and hydrate two sections to distilled water.
2. (a) Treat one section with deoxyribonuclease solution for 2 hours at 37 °C.
   (b) Treat the second section in Tris buffer solution for 2 hours at 37 ° C.
3. Remove both sections and wash together in running water for 10 minutes.
4. Stain as desired.

RESULTS. The material staining in the section incubated in Tris buffer solution and not present in the section treated with deoxyribonuclease solution is presumably *deoxyribonucleic acid.*

REFERENCE. Lillie, R. D.: *Histopathologic Technic and Practical Histochemistry,* 3rd ed., New York, McGraw-Hill Book Co., 1965, p. 147.

RIBONUCLEASE DIGESTION METHOD

FIXATION. 10% buffered neutral formalin, Carnoy's or Bouins. (Fixatives containing mercuric, ebromíc, or other heavy metal ions cannot be used).

TECHNIQUE. Cut paraffin sections 6 microns.

SOLUTIONS

RIBONUCLEASE SOLUTION

Ribonuclease, crystalline	0.5 gm
Distilled water, pH 6.8	100.0 ml

Adjust pH with 0.1N sodium hydroxide or hydrochloric acid.

DIGESTION PROCEDURE. Run two control sections.

1. Deparaffinize and hydrate two sections to distilled water.
2. (a) Treat one section in ribonuclease solution for 3 hours at 37 ° C.
   (b) Treat the second section in distilled water for 3 hours at 37 'C.
3. Remove both sections and wash together in running water for 10 minutes.
4. Stain as desired.

RESULTS. The material staining in the sections incubated in distilled water and not present in the section treated with ribonuclease solution is presumably *ribonucleic acid.*

REFERENCE. McManus, J. F. A., and Mowry, R. W.: *staining Methods Histologic and Histochemical,* New York, Hoeber Medical. Division, Harper and Row, Publishers, 1963, p. 80.

GUARD'S METHOD FOR SEX CHROMATIN

FIXATION. Fix immediately in 95% alccohol.

TECHNIQUE. Smears (buccal epithelium).

SOLUTIONS

BIEBRICH SCARLET SOLUTION

Biebrich scarlet, water soluble ..... 1.0 gm
Phosphotungstic acid, C.P .....0.3 gm
Glacial acetic acid .....5.0 ml
Alcohol, 50% ..... 100.0 ml

FAST GREEN SOLUTION

Fast green FCF (Harleco) ..... 0.5 gm
Phosphomolybdic acid .....0.3 gm
Phosphotungstic acid .....0.3 gm
Glacial acetic acid ..... 5.0 ml
Alcohol, 50% ..... 100.0 ml

HARRIS' HEMATOXYLIN SOLUTION

Harris' hematoxylin .....0.5 ml
Alcohol, 50% ..... 100.0 ml

STAINING PROCEDURE — Technique #1

I. From 95% alcohol fixative transfer to 70% alcohol for 2 minutes.
2. Biebrich scarlet solution for 2 minutes.
3. Rinse **in** 50% alcohol.
4. Differentiate in Fast green solution for 1 to 4 hours ***(See Note).***
5. Place in 50% alcohol for 5 minutes.
6. Dehydrate in 70 %, 95%, and absolute alcohol, 2 minutes each.
7. Clear in three changes of xylene, 2 minutes each.
8. Mount with Permount or Histoclad.

RESULTS — Technique #1

Sex Chromatin (Barr body) - red
Background - green

Note: During this step, check the differentiation under a microscope at hourly intervala. When all the cells reveal green cytoplasm and the vesicular nuclei are also green, the reaction is complete, which is usually in approximately 4 hours. The pyknotic nuclei, however, will not differentiate and will reveal bright red color.

STAINING PROCEDURE — Technique #2

1. From 95% alcohol fixative transfer to 70% alcohol for 2 minutes.
2. Hematoxylin solution for 15 seconds.
3. Biebrich scarlet solution for 2 minutes.
4. Place in 50% alcohol for 5 minutes.
5. Differentiate in fast green solution for 24 hours.
6. Dehydrate, olear and mount as in Technique #1.

RESULTS — Technique #2

Sex chromatin (Barr body) - red
Nuclear chromatin - blue
Cytoplasm - green

REFERENCE. Guard, H. R: ***Amer.*** *J. Clin. Path.* 32:145 -151, 1959. Copyright by Williams and Wilkins Co.

KLINGER-LUDWIG METHOD FOR SEX CHROMATIN

FIXATION. Equal parts of 95% alcohol and ethyl ether.

TECHNIQUE. Smears of buccal mucosal. cells.

SOLUTIONS

NORMAL HYDROCHLORIC ACID

(See page 158)

THIONIN SOLUTION (STOCK)

Thionin . ..... 1.0 gm
Alcohol, 50% ..... 100.0 ml

BUFFER SOLUTION (STOCK)

Sodium acetate ..... 9.714 gm
Sodium barbituate ..... 14.714 gm
Distilled water (CO_2 free) to make ..... 550.0 ml

THIONIN SOLUTION (WORKING)

Hydrochloric acid, 1N ..... 32.0 ml
Buffer solution (stock) ..... 28.0 ml
Thionin solution (stock) ..... 40.0 ml

STAINING PROCEDURE

1. Obtain relatively thick smears of buccal mucosal cells on albuminized slides.
2. Fix smears *promptly* in equal parts of 95% alcohol and ethyl ether for 15 minutes.
3. Absolute alcohol for 3 minutes.
4. Immerse in 0.2% parloidin made in equal parts absolute alcohol and ethyl ether for 2 minutes.
5. Air dry for 15 seconds.
6. 70% alcohol for 5 minutes.
7. Distilled water, two changes, for 5 minutes each.
S. Hydrolyse in normal hydrochloric acid solution at 56 °C for 5 minutes.
9. Distilled water, two changes, 5 minutes each.
10. Working thionin solution for 5 minutes.
11. Differentiate in 70% alcohol, 95% alcohol, and absolute alcohol for 1 minute each.
12. Clear in xylene.
13. Mount with Permount or Histoclad.

Sex chromatin (Barr body) - purplish red Other cell structures - light blue

REFERENCE. Klinger, H. P. and Ludwig, K. *S.: Stain Techn. 32:235, 1957.*
*Copy*right by Williams and Wilkins Co.

-1,11 SOLUTIONS

0.5% 011 RED O SOLUTION

Oil red 0 ..... 0.5 gm
Propylene glycol, 100% ..... 100.0 ml

Add a small amount of propylene glycol to the oil red O and mix well, crush largar pieces. Gradually add the remainder of the propylene glycol stirring periodically. Heat gently until the solution reaches 95 °C. Do not allow to go ovar 100 °C. Stir while heating. Filter through coarse filter papar while still warm. Allow to stand overnight at room temperatura. Filter through Seitz filter with the aid of vacuum. If solution becomes turbid, refilter. *When using the Seitz filter, put rough surface of filter up.*

85% PROPYLENE GLYCOL SOLUTION

Propylene glycol, 100% ..... 85.0 ml
Distilled water ..... 15.0 ml

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

5% ACID WATER SOLUTION

Hydrochloric acid ..... 5.0 ml
Distilled water ..... 95.0 ml

GLYCERIN JELLY

Gelatin ..... 10.0 gm
Distilled water ..... 60.0 ml
Heat until gelatin is dissolved. Add:
Glycerin ..... 70.0 ml
Phenol ..... 1.0 ml

STAINING PROCEDURE

1. Cut frozen sections and collect in distilled water.
2. Absoluta propylene glycol for 2 minutes.
3. Oil red O solution for 1 hour. *Note. If* sections are mounted on glass slides bafore staining, allow to stand in ORO overnight.
4. Differentiate in 85% propylene glycol solution for 1 minute.
5. Rinse in distilled water, two changes.
6. Stain in Mayer's or Harris' hematoxylin solution for few seconds.

RESULTS

Fat - red
Nuclei - blue

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D. C. 20305.

FISCHLER'S METHOD FOR FATTY ACIDS

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Frozen sections.

SOLUTIONS

ALCOHOL-ETHYL ETHER EXTRACTION SOLUTION

Alcohol, 100 %..... 50.0 ml
Ethyl ether ..... 50.0 ml

SATURATED CUPRIC ACETATE SOLUTION

Cupric acetate, normal ($Cu(CH_3COO)_2 \cdot H$ 10-12 gm

Distilled water ..... 100.0 ml

WEIGERT'S LITHIUM HEMATOXYLIN SOLUTION

Hematoxylin ..... 1.0 gm
Alcohol 100% ..... 10.0 ml
Dissolve hematoxylin then add:
Distilled water ..... 90.0 ml
Lithium carbonate, saturated aqueous ..... 1.0 ml

WEIGERT'S BORAX-FERRICYANIDE SOLUTION

Borax ..... 2.0 gm

Potassium ferricyanide ..... 2.5 gm
Distilled water ..... 100.0 ml

For use diluto one part of this solution with 10 parts of distilled water. **STAINING PROCEDURE.** Run two control sections. Treat one section as follows:

EXTRACTION TEST

1. Dehydrate through 95%, and absoluto alcohol, two changes each.
2. Placo in alcohol-ethyl ether extraction solution for three changes, 2 minutes each.

Treat both sections as follows:

4. Cupric acetate solution for 2 - 24 hours at room temperatura.

o

8. Rinse well in distilled water.

9. Mount with glycerin jelly.

RESULTS

Fatty acids - deep blue-black

Irou, hemoglobin and calcium may also stain and are not removed by the extraction procedure. Free fatty acid will be removed by the extraction procedure.

ote. Following the extraction test, only the calcium soaps will remain.

REFERENCE. Fischler. F.: Ober die Unterscheidung von Neutralfetten, Fettsãuren und Seifen im Gewebe, *Zbl. Allg. Path. 15:913, 1904.*

OSMIUM TETROXIDE METHOD FOR FAT (FROZEN SECTIONS)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Frozen sections.

SOLUTIONS
fat.

6. Rinse well in distilled water.

7. Mount with glycerin jelly.

RESULTS

at - black

TECHNI QUE. Cutparaffin sectionsat6 microns. *Note.* (SeestepI,stainingprocedure).

SOLUTIONS

5% POTASSIUM DICHROMATE SOLUTION (STOCK)

Potassium dichromate .....5.0 gm

Distilled water ..... 100.0 ml

2% OSMIUM TETROXIDE SOLUTION (STOCK)

Osmium tetroxide ..... 2.0 gm

STAINING PROCEDURE

4. Place formalin-fixed specimen, which is no thicker than 4 mm, in working potassium dichromate-osmium tetroxide solution for 8 hours.

5. Wash specimen in running water for 2 hours.

6. Process overnight in conventional automatic tissue processos. Embed and cut sections at 6 microns.

7. Deparaffinize sections and hydrate to distilled water.

8. Counterstain with hematoxylin and eosin or kernechrot.

9. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

10. Mount with Permount or Histoclad.

RESULTS

Fat - black

REMARKS. The following modification to the previous method has proveu extremely valuable in our laboratories since it eliminates the many artifacts created in frozen section cutting and/or staining. This method utilizes the fat-preserving qualities of osmium tetroxide and the staining qualities of oil red O. Using the following procedure, we have been atile to demonstrate small intracytoplasmic fat droplets not visible with conventional fat stams:

Prepare tissues as above through Step 4. At this point remove osmium tetroxide with a 3% hydrogen peroxide (normally 4 hours is adequate) followed by a 15 minute wash in tap water. Sections are then stained in oil red O for 1 hour. Wash in water and mount in glycerin jelly.

Note. It may be necessary to use a 5% aqueous periodic acid solution for 5 minutes following Step 4 to insure section background is olear enough to accept the counterstain desired.

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C., 20305.

SUDAN BLACK B METHOD FOR FAT

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Frozen sections.

SOLUTIONS

SUDAN BLACK B SOLUTION

Dissolve 0.7 gm Sudan black B in 100 ml of propylene glycol by heating to 100' — 110 °C and stirring thoroughly for a few minutes. Do not exceed 110 °C. Filter hot through Whatman No. 2 papar to remove excess dye. After cooling to room temperatura filter through a Seitz filter with the aid of vacuum.

85% PROPYLENE GLYCOL SOLUTION

(See page 141)

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

2. Cut frozen sections and collect in distilled water.
3. Absoluto propylene glycol for 10 minutes.
4. Sudan black B solution for 30 minutes. *Note.* If sections are mounted on glass slides bafore staining, allow to stain in Sudan black B overnight.
5. Differentiate in 85% propylene glycol solution for 3 minutes. Agitate sections several times.
6. Rinse in distilled water.
7. Counterstain in nuclear fast red for 5 minutes.
8. Rinse well in distilled water.
S. Mount with glycerin jelly.

RESULTS

Fat - blue-black
Nuclei - red

REFERENCE. Chiffelle, T.L., and Putt, F.A.: *Stain Techn.* 26:51, 1951.

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LANDINGS METHOD FOR LIPIDS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns. Cut frozen sections at 10-12 microns.

SOLUTIONS

ACETONE-ETHYL ETHER SOLUTION

Acetone ..... 50.0 ml
Ethyl ether ..... 50.0 ml

PHOSPHOMOLYBDIC ACID SOLUTION

Phosphomolybdic acid ..... 1.0 gm

Alcohol 100% ..... 50.0 ml
Chloroform ..... 50.0 ml

STANNOUS CHLORIDE SOLUTION

Stannous chloride ..... 1.0 gm

Hydrochloric acid, 3N ..... 100.0 ml

Make this solution fresh just before use.

3N HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, concentrated ..... 10.0 ml

RESULTS

Gaucher lipid (kerasin)	–deep blue -lighter blue green
Niemann-Pick lipid (sphingomyelin)	– deep blue
Tay-Sachs' (ganglion cells)	– deep blue
Normal myelin	

REFERENCE. Landing, B. H., Uzman, L. L., and Whipple, A.: Lab. Invest. 1:456 - 462, 1952.

BAKER'S METHOD FOR PHOSPHOLIPIDS

FIXATION. Baker's formol calcium fixative or 10% buffered neutral formalin.

TECHNIQUE. Frozen sections.

SOLUTIONS

POTASSIUM DICHROMATE -CALCIUM SOLUTION

Potassium dichromate ..... 5.0 gm

calcium chloride ..... 1.0 gm

Distilled water ..... 100.0 ml

BORAX-FERRICYANIDE SOLUTION

Borax ..... 0.25 gm

Potassium ferricyanide ..... 0.25 gm
Distilled water ..... 100.0 ml

Store in refrigerator.

RESULTS

ospholipids (lecithin, kephalin, sphingomyelin) - blue black

ılactolipids - blue-black to pala blue

PYRIDINE EXTRACTION TEST

Pyridine extraction test is designed to distinguiste between phospholipids and certain proteins which give a positive reaction with the acid hematein procedure.

SOLUTIONS

WEAK BOUIN'S SOLUTION

Picric acid, saturated aqueous ..... 50.0 ml

Formalin, 37 — 40% ..... 10.0 ml
Glacial acetic acid ..... 5.0 ml

-101) Luxol fast blue MBS may be substituted for Methasol fast Blue 2G.

0.05% LITHIUM CARBONATE SOLUTION

Lithium carbonate ..... 0.05 gm
Distilled water ..... 100.0 ml

1% NEUTRAL RED SOLUTION

Neutral red ..... 1.0 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and place in absolute alcohol.
2. Methasol fast blue solution at *58 °C* overnight.
3. Rinse in 70% alcohol.
4. Rinse in distilled water.
5. Differentiate in lithium carbonate solution for 30 minutes to 2 hours.
6. Rinse in distilled water.
7. Counterstain in neutral red solution for 30 minutes.
8. Rinse in distilled water.
10. Blot dry.
11. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes, 2 minutes each.
12. Mount with Permount or Histoclad.

RESULTS

Phospholipids	- blue
Early lipofuchsin, eosinophil granules, keratin, keratohyaline, human elastic tissues	- blue to purple
Nuclei and nucleoli	-red

REFERENCE. Pearse, A. G. E.: *J. Path. Bact. 70:554 - 557, 1955.*

~HERAS HISTOCHEMICAL METHOD FOR UNSATURATED LIPIDS

FIXATION. 10% Buffered neutral formalin.

TECHNIQUE. Frozen sections.

SOLUTIONS

3.5% BROMINE SOLUTION

Bromine' ..... 3.5 gm
Distilled water ..... 100.0 ml

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See pane *89)*

10% SILVER NITRATE SOLUTION

Silver nitrate . ..... 10.0 gm
Alcohol, 50% aldehyde free ..... 100.0 ml

REDUCING SOLUTION

Formalin, 37 — 40%..... 10.0 ml
Distilled water ..... 90.0 ml

STAINING PROCEDO RE. Use control slide*.

1. Cut frozen sections and collect in distilled water.
2. Wash in tap water for several hours followed by several changes of distilled water.
3. Blot dry.
4. Expose to Bromine solution fumes in a large closed jar at 37 'C for 1 hour.
5. Sodium thiosulfate solution, two changes, 5 minutes each.
6. Rinse in distilled water, three changes.
7. Silver nitrate solution at 37 'C in the dark until faint brown or pale yellow.
8. Rinse in distilled water.
9. Place in reducing solution until deep brown.
10. Rinse in distilled water.
11. Sodium thiosulfate solution for 2 minutes.
12. Rinse in distilled water.
13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes, 2 minutes each.
14. Mount with Permount or Histoclad.

RESULTS

Unsaturated lipids brown to black

REMARKS. If a criticai study of unsaturated lipid is desired, sections should be treated with amylase before bromination after step 2 to eliminate any glycogen interfering with the unsaturated lipid.

REFERENCE. Mukherji, M., Deb, C., and Sen, P. B.: *J. Histochem. Cytochem.* 8:189 -194, 1960. Copyright by Williams and Wilkins Co.

SELIGMAN-ASHBEL METHOD FOR ACTIVE CARBONYL GROUPS

FIXATION. 10% buffered neutral. formalin (not more than 48 hours).

TECHNIQUE. Cut frozen sections at 15 microns.

SOLUTIONS

0.1% 2-HYDROXY-3-NAPHTHOIC ACID HYDRAZIDE* SOLUTION

2 - hydroxy - 3 - napthoic acid hydrazide ..... 0.1 gm

Dissolve powder in hot glacial acetic acid then add:

Alcohol, 50% 95.0 ml

5% ACETIC-ALCOHOL SOLUTION

Glacial acetic acid ..... 5.0 ml
Alcohol, 50% ..... 95.0 ml

0.67M PHOSPHATE BUFFER SOLUTION, pH 7.4

Y15 M sodium phosphate, dibasic (9.465 gm made up to 1000 ml with distilled water) ..... 80.0 ml

S5 *M* potassium acid phosphate (9.07 gm made up to 1000 ml with distilled water) ..... 20.0 ml

PHOSPHATE BUFFER-ALCOHOL SOLUTION

Phosphate buffer solution ..... 50.0 ml
Alcohol, 100% ..... 50.0 ml

FAST BLACK B SOLUTION

Fast black B ..... 0.05 gm.
Phosphate buffer-alcohol solution ..... 100.0 ml

0.1% SAFRANIN SOLUTION

Safranin ..... 0.1 gm
Glacial acetic acid, 1% aqueous ..... 100.0 ml

STAINING PROCEDURE. Mount sections on glass slides and let dry in 37°C oven overnight.

1. Wash slide in running water for 2 hours to remove formalin.
2. Place in fresh naphthoic-hydrazide solution for 2 hours at 25 ° C.
3. Wash in acetic-alcohol solution, 50% alcohol, and distilled water for four changes, 2 minutes each.
4. Phosphate buffer-alcohol solution for 1 -2 minutes.
5. Directly finto fast black B solution for 2 minutes.
6. Rinse in acetic-alcohol solution.
7. Counterstain with safranin solution for 1- 2 minutes.
8. Rinse in distilled water.
9. Mount with glycerin jelly.

RESULTS. Active carbonyl lipids -dark blue or greenish blue.

REFERENCE. Seligman, A. M., and Ashbel, *R.:Endocrinology* 50:338 -349, 1952.

*K & K Laboratories, 121 Express Street, Engineers Hill, Plainview, New York, 11803.

MENSCHIK'S METHOD FOR PHOSPHOLIPIDS

FIXATION. Baker's formol calcium.

TECHNIQUE. Frozen sections.

SOLUTIONS

NILE BLUE SULPHATE SOLUTION

Nile blue sulphate, saturated aqueous ..... 100.0 ml

Sulphuric acid, 0.05% aqueous ..... 10.0 ml

Boil for 30 minutes using a reflui condensei.

5% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid ..... 5.0 ml

Distilled water ..... 95.0 ml

0.5% HYDROCHLORIC ACID SOLUTION

Hydrochloric acid, concentrated ..... 0.5 ml

Distilled water ..... 99.5 ml

GLYCERIN JELLY

(See page 141) **STAINING**

Chapter 10

Methods For Carbohydrates And Mucoproteins

BENNHOLD'5 METHOD FOR AMYLOID (CONGO RED)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns (see Remarks).

SOLUTIONS

1% CONGO RED SOLUTION

Congo red ..... 1.0 gm

Distilled water ..... 100.0 ml

1% SODIUM HYDROXIDE SOLUTION

Sodium hydroxide ..... 1.0 gm

REMARKS. Sections of suspected amyloidosis may be cut at 12 microns if a brighter color is desired. This thick section technic is also being used to advantage in our laboratories to demonstrate minute amyloid deposits. Twelve mícron sections can also be used to advantage in the crystal violet and Sirius Red methods for amyloid.

REFERENCE. Mallory, F. B.: Pathological Technique, New York, Hafner Publishing Co., 1961, p. 133 -134. (AFIP modification)

LIEB'S METHOD FOR AMYLOID (CRYSTAL VIOLET)

FIXATION. 10% buffered neutral formalin or alcohol.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CRYSTAL VIOLET SOLUTION (STOCK)

Crystal violet to saturate ..... approx. 14.0 gm

Alcohol, 95% ..... 100.0 ml

CRYSTAL VIOLET SOLUTION (WORKING)

Crystal violet (stock) ..... 10.0 ml

Distilled water .....300.0 ml

SOLUTIONS

1% SODIUM HYDROXIDE SOLUTION

(See page 153)

~ALINE ALCOHOL SOLUTION

Alcohol, 80% ..... 100.0 ml

Sodium hydroxide, 1 % ..... 1.0 ml

SIRIÚS RED SOLUTION

Sirius Red, F3BA' ..... 1.0 gm

Distilled water ..... 100.0 ml

issolve and then add:

Sodium chloride ..... 0.5 gm

Let stand overnight before use. *DO NOT FILTER.*

UM BORATE BUFFER SOLUTION pH 9.0

REMARKS. Sweat and Puchtler used seven direct cotton dyes for the demonstration of amyloid. In studies conducted in our laboratories Sirius Red F3BA has been found to be the most specific. (See remarks, Bennhold's method).

Note. Sodium borate ($^{Na}2B407\ -^{10H}20$) must be used as other borates will give a different pH.

REFERENCE. Sweat, F. and Puchtler, H.: *Arch. Path.* 80:613 - 620, 1965.

HIGHMAN'S METHOD FOR AMYLOID (METHYL VIOLET)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See paga 35)

0.1% METHYL VIOLET SOLUTION

Methyl violet ..... 0.1 gm

Distilled water ..... 97.5 ml

Glacial acetic acid ..... 2.5 ml

APATHY'S MOUNTING MEDIA

Acacia (gum arabic) ..... 50.0 gm

Cana sugar (sucrose) ..... 50.0 gm

Distilled water ..... 150.0 ml

Sodium chloride ..... 10.0 gm

Thymol.....0.1 gm

Mix the acacia, cave sugar, and distilled water in a flask. Placa the flask in a pan of boiling water until ingrediente are dissolved. Dissolve the sodium chloride and thymol in this solution. Filter with a Seitz filter while the solution is still warm, or use coarse filter paper and placa in 60°C oven, changing filter paper frequently. Refrigerate to remove air bubbles. Sodium chloride is added to prevent bleeding and thymol is added as a preservativa.

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Working Weigert's hematoxylin solution for 5 minutes.
3. Wash in running water for 10 minutes.

VASSAR-CULLING METHOD FOR AMYLOID (THIOFLAVINE T)

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% THIOFLAVINE T SOLUTION

Thioflavin T[O] ..... 1.0 gm

Distilled water ..... 100.0 ml

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

DIFFERENTIATING SOLUTION

Glacial acetic acid ..... 1.0 ml

Distilled water ..... 100.0 ml

APATHY'S MOUNTING MEDIA

(See page 156)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 5 minutes. Do not differentiate.
3. Wash in running water for 1 minute.
4. Thioflavine T solution for 3 minutes.
5. Wash in running water for 10 minutes.
6. Differentiating solution for 10 minutes.
7. Wash in running water for 2 minutes.
8. Mount in Apathy's mounting media.

RESULTS

Amyloid white fluorescente

Note. Since this paper was published the authors (P.S.V. - C.F.A.C.) have found that increased specificity of staining is achieved by using a shorter wave length of light (Zeiss UG5 excitor filter) and a simple (colorless) U.V. filter in the eyepiece. Using this system only Amyloid is yellow, all other tissue constituents are blue.

REFERENCE. Vassar, P. S. and Culling, C. F. *A.:Arch. Path.* 68:487-498, 1959.

CARMINE SOLUTION (STOCK)

Carmine ..... 2.0 gm

Potassium carbonate ..... 1.0 gm

Potassium chloride ..... 5.0 gm

Distilled water ..... 60.0 ml

Boil in an evaporating dish gently and cautiously for several minutes. When cool add 20 ml of 28% ammonium hydroxide. Store in refrigerator.

CARMINE SOLUTION (WORKING)

Carmine solution (stock) ..... 10.0 ml

Ammonium hydroxide, 28% ..... 15.0 ml

Alcohol, methyl. ..... 15.0 ml

DIFFERENTIATING SOLUTION

Alcohol, 100% ..... 20.0 ml

Alcohol, methyl ..... 10.0 ml

Distilled water ..... 25.0 ml

STAINING PROCEDURE. For digestion procedure, see page 171.

2. Deparaffinize and hydrate to distilled water.
3. Mayer's hematoxylin solution for 15 minutes.
4. Wash in running water for 15 minutes.
5. Working carmine solution for 30 minutes.
6. Differentiating solution for a few seconds.
7. Rinse quickly in 80% alcohol.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

S. Mount with Permount or Histoclad.

RESULTS

Glycogen - pink to red

Nuclei - blue

COLEMAN'S FEULGEN SOLUTION

Dissolve 1.0 gm basic fuchsin in *200.0* ml hot distilled water. Bring to boiling point. Cool and add *2.0* gm potassium metabisulfite, 10.0 ml normal hydrochloric acid. Let bleach for *24* hours, then add *0.5* gm activated carbon (Norit). Shake for 1 minute and filter through coarse filter paper. Repeat filtration until solution is colorless. *Store in refrigerator.*

SCHIFF REAGENT SOLUTION

Dissolve 1.0 gm basic fuchsin in 200.0 ml hot distilled water. Bring to boiling point. Cool to *50 °C.* Filter and add 20.0 ml normal hydrochloric acid. Cool further and add 1.0 gm anhydrous sodium bisulfite, or sodium metabisulfite. Keep in the dark for *48* hours until solution becomes straw colored. *Store in refrigerator.*

Test For Schiff Reagent Solution

Pour a few drops of Schiff reagent solution finto 10 ml of *37 — 40%* formaldehyde in a watch glass. If the solution turns reddish purple rapidly, it is good. If the reaction is delayed and the resulting color deep blue-purple, the solution is breaking down.

0.5% PERIODIC ACID SOLUTION

Periodic acid	0.5 gm
Distilled water	100.0 ml

0.2% LIGHT GREEN SOLUTION (STOCK)

Light green, SF yellowish	0.2 gm
Distilled water	100.0 ml
Glacial acetic acid	0.2 ml

LIGHT GREEN SOLUTION (WORKING)

Light green (stock)	10.0 MI.
Distilled water	50.0 ml

HARRIS' HEMATOXYLIN SOLUTION

(See page *34)*

STAINING PROCEDURE. For digestion procedure see page *171.*

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in periodic acid solution for *5* minutes.
3. Rinse in distilled water.
4. Coleman's Feulgen *or* Schiff reagent solution for *15* minutes.
5. Wash in running water for 10 minutes for pink color to develop.
6. Harris' hematoxylin for 6 minutes, *or* light green couriterstain for a few seconds. Light green is recommended for counterstaining sections in which fungi are to be demonstrated. Omit steps *7* through 11 if light green is used.
7. Wash in running water.
8. Differentiate in 1% (HCL) acid alcohol ~ three to ten quick dips.
9. Wash in running water.
10. Dip in ammonia water to blue sections.

11. Wash in running water for 10 minutes.
12. Dehydrate in 95% alcohol, absoluta alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Glycogen, mucin, reticulin, fibrin or thrombi, colloid droplets, hyalin of arteriosclerоses, hyalin deposits in glomeruli, granular cells in the renal arterioles where preserved, most basement membranas, colloid of pituitary stalks and thyroid, amyloid infiltration may show a positive reaction - rose to purplish red

Nuclei - blue
Fungi - red
Background - pala green (with light green counterstaining).

REMARKS. For excellent additional information on colloidal. iron, Alcian blue 8GX and their combinations with periodic acid Schiff reaction see: "The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins with revised directions for the colloidal iron stain, the use of alcian blue 8GX and their combinations with the periodic acid-Schiff reaction," Mowry, *R. W. Annals of the New York* ***Academy*** *of Sciences,* 106:402 -423, 1963.

Note: A solution of 5% aqueous Clorox bleach will reduce overstaining by leucofuchsin. Running tap water or ammonia water will decolorize the light green. REFERENCE. McManus, J. F. *A.: Stain Techn.* 23:99 - 108, 1948. (AFIP modification).

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LAQUEUR'S METHOD FOR ALCOHOLIC HYALIN

FIXATION. Zenker's or 10% buffered neutral formalin. TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

ACID FUCHSIN-ANILINE SOLUTION

Acid fuchsin ..... 20.0 gm
Anilina ..... 2.0 ml
Distilled water ..... 100.0 ml

Shake the anilina in distilled water thoroughly bafore adding the acid fuchsin.

ALCOHOLIC PICRIC ACID SOLUTION

Picric acid, saturated in 100% alcohol ..... 10.0 ml
Alcohol, 20% ..... 70.0 ml

r141W

1% PHOSPHOMOLYBDIC ACID SOLUTION

(See page 112)

1% LIGHT GREEN SOLUTION

Light green, SF yellowish ..... 1.0 gm

Distilled water ..... 100.0 ml

Glacial acetic acid ..... 1.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Mayer's hematoxylin solution for *15* minutes.

3. Wash in running water for *15* minutes.

4. Flood the slides with acid fuchsin-aniline solution and heat gently with bunsen burner until aniline fumes escape, let stand for *5* minutes.

5. Wash in water, three dips.

6. Differentiate in alcoholic picric acid solution until only

Mix stain in small evaporating dish. Heat on electric hot plate for 2 minutes. Liquid becomes black 'and syrupy. Dilute with 100 ml of 50% alcohol and let stand for 24 hours. Filter.

Dilute 1 part mucicarmine solution with 4 parts *tap water* for use.

0.25% METANIL YELLOW SOLUTION

(See page 78)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Working solution of Weigert's hematoxylin for 7 minutes.

3. Wash in running water for 10 minutes.

4. Diluted mucicarmine solution for 60 minutes.

3. Toluidine blue solution for 2 minutes.

4. Rinse in distilled water.

5. Cover slip section with distilled water.

6. Blot around edge of cover glass and seal with fingernail polish.

7. Examine the sections as soou as possible after preparation.

RESULTS

Metachromatic tissue - pink

REFERENCE. Johnson, F. B.: Histochemistry Branch, Armed Forces Instituto of Pathology, Washington, D. C. 20305.

ALCIAN BLUE METHOD FOR MUCOSUBSTANCES pH 2.5 or pH 1.0

Alcian Blue pH 1.0 Method

SOLUTIONS

ALCIAN BLUE SOLUTION

Alcian Blue, 8GX ..... 1.0 gm

Hydrochloric acid, 0.1N ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Alcian blue solution for 30 minutes.

3. Blot sections dry with filter papar without rinsing in water.

4. Dehydrate in *95%* alcohol, absoluta alcohol, and clear in xylene, two changes each.

5. Mount with Permount or Histoclad.

RESULTS. This method providas a more selectiva staining of sulfated mucosubstances which stain dark blue.

REFERENCE. Lev, R. and Spicer, S. S.: *J. Histochem. Gytochem.* 12:309, *1964.* *Copy*-right by Williams and Wilkins Co.

ALCIAN BLUE METHOD FOR MUCOSUBSTANCES pH 0.4

S. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

9. Mount with Permount or Histoclad.

RESULTS. Only strongly acidic sulfated muco substances give a positive reaction.

REFERENCE. Johnson, W. C., Graham, J. H., and Helwig, E. B.: *J. Invest. Derm.* 42:215 -224, 1964.

ALDEHYDE FUCHSIN METHODS FOR MUCOSUBSTANCES

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

ALDEHYDE FUCHSIN SOLUTION pH 1.7

(See pape 166)

STAINING PROCEDURE. For digestion procedures, see papes 171, 172.

2. Deparaffinize and hydrate to distilled water.
3. Rinse in 709%, alcohol.
4. Aldehyde fuchsin solution for 30 minutes.
5. Rinse in 70/1',, alcohol.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Sulfated mucosubstances	-dark purple
Elastica	- dark purple
Mast cells	- dark purple
Sialomucins and hyaluronic acid	- f aint reaction
Paget's cells	- purple

ALDEHYDE FUCHSIN -ALCIAN BLUE METHOD

SOLUTIONS

ALCIAN BLUE SOLUTION

Alcian blue, 8GX	0.2 gm
Glacial acetic acid, 3/1"/o aqueous	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Rinse in 70% alcohol.
3. Aldehyde fuchsin solution for 30 minutes.
4. Rinse in 70% alcohol, rinse in distilled water, several changes, for 5 minutes.

6. Alcian blue solution for 30 minutes.

7. Rinse- in distilled water.

8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

9. Mount with Permount or Histoclad.

RESULTS

Strongly acidic sulfated mucosubstances and elastica	- deep purple
Less strongly acidic sulfated mucosaccharide or mixed sulfated	
nd carboxylic mucosubstances	- blue to purple
onsulfated acid mucosubstances	- blue

REFERENCE. Spicer, S. S. and Meyer, D. B.: *Techn. Bull. Regist. Med. Techn.* 30:53 - 60, 1960. Copyright by Williams and Wilkins Co.

ALDEHYDE FUCHSIN pH 1.0

FIXATION. 10% buffered neutral formalin or fixative of choice.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

RESULTS. All mucins except the highly acidic sulfated mucosubstances are aldehyde-fuchsin negative at pH values of 1.0 and below.

REFERENCE. Johnson, W. C., Graham, J. H and Helwig, E. B.: *J. Invest. Derm.* 42:215 - 224, 1964.

MODIFICATION OF MOWRY'S 1958 COLLOIDAL IRON STAIN FOR ACID MUCOPOLYSACCHARIDES

FIXATION. 10% buffered neutral formalin or 95% alcohol. Bichromate fixatives should be avoided.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

29% FERRIC CHLORIDE SOLUTION

Ferric chloride ..... 29.0 gm

Distilled water ..... 100.0 ml

MÜLLER'S COLLOIDAL IRON OXIDE SOLUTION (STOCK)

Bring 250 ml of distilled water to a boil. While the water is still boiling, pour 4.4 ml of 29% ferric chloride solution (USP XI) and stir. When the solution has turned dark red, remove froco heat and allow to cool. *Label: Stock Müller's colloidal Irou.* This reagent is stable for many months. In tbe staining procedures, the stock solution of colloidal iron is diluted just before use as follows:

COLLOIDAL IRON SOLUTION (WORKING)

Müller's colloidal iron (stock) ..... 20.0 ml

VAN GIESON SOLUTION

(See page 76)

STAINING PROCEDURE. Use control, slide. For digestion procedures, see pages 171, 172.

1. Deparaffinize and hydrate to distilled water.
2. Rinse in glacial acetic acid solution for 3 minutes.
3. Working colloidal irou solution for 1 hour.
4. Rinse in glacial acetic acid solution for four changes, 3 minutes each.
5. Hydrochloric acid-potassium ferrocyanide solution for 20 minutes.
6. Wash in tap water for 5 minutes.
7. Rinse in distilled water.
8. Van Gieson's solution for 5 to 7 minutes.
9. Without washing, pass quickly to 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
10. Mount with Permount or Histoclad.

RESULTS

Acidic mucopolysaccharides and acidic epithelial mucins are colored deep (Prussian) blue .

Background depending on counterstain used.

REFERENCE. Mowry, R. W.: Department of Pathology, University of Alabama Medical, Center, Birmingham, Alabama 35233. (AFIP modification).

PAS -ALCIAN BLUE METHOD FOR MUCOSUBSTANCES pH 2.5 or 1.0

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% PERIODIC ACID SOLUTION

(See page 113)

SCHIFF REAGENT SOLUTION

(See page 159)

ALCIAN BLUE SOLUTION pH 2.5

(See page 163)

ALCIAN BLUE SOLUTION pH 1.0

(See page 164)

0.5% SODIUM METABISULFITE SOLUTION

(See page 72)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Alcian blue solution (pH 2.5 or 1.0) for 30 minutes.
3. Blot section dry with filter paper if Alcian blue solution pH 1.0 is used. Wash in water after Alcian blue pH 2.5 for 5 minutes.
4. Oxidize in periodic acid solution for 10 minutes.
5. Wash in running water for 5 minutes.
6. Schiff reagent solution for 10 minutes.
7. Rinse in sodium metabisulfite solution three changes, 2 minutes each.
8. Wash in running water for 10 minutes.
9. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
10. Mount with Permount or Histoclad.

RESULTS. *PAS-Alcian blue pH 2.5.* All polysaccharides and mucosubstances containing hexoses or deoxyhexoses with vicinal glycol groups stain magenta to red. Those mucosubstances staining red include neutral mucosubstances. Hyaluronic acid, sialomucins and all but the most strongly acidic sulfated mucosubstances stain blue.

PAS-Alcian blue pH 1.0. Pas positive same as above. Alcinophilic substances at this pH include only the sulfated mucosaccharides.

REFERENCE: Lev, R., Spicer, S. S.: *J. Histochem. Cytochem.* 12:309, 1964.

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SAUNDERS METHOD FOR ACID MUCO POLYSACC HARI DES

FIXATION. Newcomer's solution for 24 hours (see *Notes* for processing schedule).

TECHNIQUE. Cut paraffin sections at 6 microns (see *Notes* for ribboning technique).

SOLUTIONS

NEWCOMER'S SOLUTION

Alcohol, isopropyl ..... 60.0 ml

Propionic acid ..... 30.0 ml

ACRIDINE ORANGE SOLUTION pH 3.2

Acridine orange ..... 0.1 gm
Glacial acetic acid, *0.01 M*
(0.6 ml /1000 ml distilled water) ..... 100.0 ml

DIFFERENTIATING SOLUTION 1

Sodium chloride, 0.3 *M (17.53* gm/1000 ml
distilled water) ..... 50.0 ml
Glacial acetic acid, *0.01 M* (0.6 MI/1000 ml
distilled water) ..... 50.0 ml

DIFFERENTIATING SOLUTION II

Sodium chloride, 0.6 M (35.05 gm/1000 ml
distilled water) ..... 50.0 ml
Glacial acetic acid, *0.01 M (0.6* m1/1000 ml
distilled water) ..... 50.0 ml

FLUORMOUNT* (FLUOR-FREE MOUNTING MEDIA)

NING PROCEDURE. Run three comparable sections (see step 5).

1. Deparaffinize and hydrate to distilled water.

2. Cetyltrimethylammonium chloride solution for *3* minutes.

3. Wash in tap water for 10 minutes.
4. Ribonuclease solution at *45* °C for *2* hours.

5. Slide 1 is again treated for *3* minutes with cetyltrimethylammonium chloride, washed 10 minutes in running water, stained *3* minutes in acridine orange pH *7.2* solution, washed in running water for 10 minutes, air dried and mounted with Fluormount. *Slide 2 is* stained *5* minutes in acridine orange pH *3.2,* washed in running water for *5* minutes, placed in differentiating solution I for *5* minutes, air dried and mounted with Fluormount. *Slide 3* is treated as slide *2* but placed in differentiating solution 11 then air dried and mounted with

REFERENCE. Saunders, A. M.: *J. Histochem. Cytochem.* 12:164 -170, 1964. Copyright by Williams and Wilkins Co.

DIASTASE DIGESTION METHOD

PHOSPHATE BUFFER SOLUTION pH 6.0

Sodium chloride ..... 8.0 gm

Sodium phosphate, dibasic ..... 0.282 gm

Sodium phosphate, monobasic..... 1.97 gm

Distilled water ..... 100.0 ml

DIASTASE DIGESTION SOLUTION

Diastase of malt ..... 0.1 gm

Phosphate buffer solution, pH 6.0 ..... 100.0 ml

Mix just before use

RESULTS. Staining attributable to hyaluronic acid, chondrotin 4 sulfate or chondrotin 6 sulfate is' selectively eliminated.

SIALIDASE DIGESTION METHOD

Use two slides from the same block. One for digestion and one for Buffer Solution 9#2 treatment.

SOLUTIONS

BUFFER SOLUTION #1

1.0 M Sodium Acetate - 0.4 M Calcium Chloride

Sodium acetate .....26.2 gm

Calcium chloride ..... 8.9 gm

Distilled water ..... 200.0 ml

BUFFER SOLUTION #2

0.1 M Sodium Acetate-0.04 M Calcium Chloride

Sodium acetate ..... 2.6 gm

Calcium chIoride..... 0.89 gm

in Lillie, R. D.: *Histopathological Technic and Practical Histochemistry,* 3rd ed., New York, McGraw-Hill Book Company, 1965.

Chitin

Inulin

Cellulose

Pectin

Starch

Dextran

Lignin

Corpora amylacea

Chapter 11

Methods For Pigments And Minerais

HALL'S METHOD FOR BILIRUBIN

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

FOUCHET'S REAGENT

Trichloroacetic acid ..... 25.0 gm
Distilled water ..... 100.0 ml
Mix and add:

10% Ferric chloride ..... 10.0 ml

10% FERRIC CHLORIDE SOLUTION

Ferric chloride ..... 10.0 gm

CARR'S METHOD FOR CALCIUM

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at, 6 microns.

SOLUTIONS

CHLORANILIC ACID SOLUTION

Distilled water ..... 100.0 ml

Sodium hydroxide ..... 0.4 gm
Chloranilic acid ..... 1.0 gm

Agitate until all the acid is dissolved. Filter through fine filter paper.

1% LIGHT GREEN SOLUTION

Light green, SF yellowish ..... 1.0 gm

1% ALIZARIN RED S SOLUTION

Alizarin red S ..... 1.0 gm

0.1% AMMONIUM HYDROXIDE SOLUTION

Ammonium hydroxide, C. *P. 28%* ..... 0.1 ml
Distilled water ..... 100.0 ml

1% LIGHT GREEN SOLUTION

(See page 161)

STAINING PROCEDURE

1. Deparaffinize and hydrate to *95%* alcohol (drain off excess *95%* alcohol).
2. Alizarin Red S solution for *2* minutes.
3. Remove excess stain with distilled water, five or six rinses.
4. Counterstain in light green solution for 1 minute.
5. Rinse in distilled water for *5 -10* seconds.
6. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Calcium salts - intense reddish-orange
Background - pale green

REFERENCE. Dahl, L. K.: *J. Exp. Med. 95:474-479, 1952.*
Permission by The Rockefeller University Press.

KOSSA'S METHOD FOR CALCIUM

FIXATION. Absolute alcohol, formol alcohol or 10% buffered neutral formalin. Alcohol fixatives are preferred.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

5% SILVER NITRATE SOLUTION

(See page *91)*

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page *89)*

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page *89)*

STAINING PROCEDURE. Use control slide. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water.
2. Silver nitrate solution for 60 minutes exposed to direct sunlight, ultra-violet lamp, or a 100-watt lamp light.
3. Rinse in distilled water.
4. Sodium thiosulfate solution for 2 minutes.
5. Rinse well in distilled water.
6. Counterstain in nuclear fast red solution for 5 minutes.

7. Rinse in distilled water.

S. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

9. Mount with Permount or Histoclad.

RESULTS

Calcium salts - black
Nuclei -red
Cytoplasm - light pink

REMARKS. Although the use of ultra-violet lamp and a 100-watt bulb for developing the silver is suggested, we strongly recommend direct sunlight be used whenever possible.

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 144.

PIZZOLATO'S METHOD FOR CALCIUM OXALATE

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SILVER N 1 TRATE -HYDROGEN PEROXIDE SOLUTION

Silver nitrate, 5% aqueous.....25.0 ml

Hydrogen peroxide, 30% .....25.0 ml

Mix just before use.

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Pour silver nitrate-hydrogen peroxide solution onto slides in sufficient quantities to cover section. Place 60-watt light bulb 6 inches froco sections for 30 minutes.
3. Rinse thoroughly with distilled water.
4. Counterstain in Kernechtrot for 5 minutes.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Calcium oxalate - black
All other tissue elements - red

Note. Numerous gas bubbles will develop during the light bulb staining treatment if a large amount of egg albumin section adhesive is present. This tends to inhibit the reaction. Should this happen, however, it will be necessary 'to pour off the mixture at this point and add fresh solution

REFERENCE. Pizzolato, P.: *J. Histochem. Cytochem. 12:333 - 336, 1964.* Copyright by Williams and Wilkins Co.

UZMAN'S METHOD FOR COPPER

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at *6* microns.

SOLUTIONS

RUBEANIC ACID (DITHIOOXAMIDE) SOLUTION

Dithiooxamide*	0.1 gm
Alcohol, 70%	100.0 ml

STAINING **PROCEDURE**

1. Deparaffinize in xylene, two changes.

2. Equal parts of xylene-absoluto alcohol, two changes.

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

(See page 89)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. Ammonium sulfide solution for 4 hours.

3. Rinse thoroughly in distilled water.

4. Hydrochloric acid-potassiurn ferricyanide solution for 20 minutes.

5. Rinse thoroughly in distilled water.
6. Counterstain in nuclear fast red solution for 5 minutes.

i

4. Counterstain in nuclear fast red solution for 5 minutes.

5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

ron pigments -bright blue

Nuclei - red

Cytoplasm - light pink

Note. Stock solutions of bathophenanthroline is stable for 4 weeks if kept in the refrigerator.

REFERENCE. Hukill, P., and Putt, A.: *J. Histochem. Cytochem.* 10:490 -494, 1962.
Copyright by Williams and Wilkins Co.

JOHNSON'S METHOD FOR IRON (MICROINCINERATION)

FIXATION. 10% buffered formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

TIONS

5% POTASSIUM FERROCYANIDE SOLUTION (STOCK)

(See page 167)

0.6N HYDROCHLORIC ACID SOLUTION (STOCK)

Hydrochloric acid, concentrated ..... 50.0 ml

Distilled water ..... 950.0 ml

HYDROCHLORIC ACID-POTASSIUM FERROCYANIDE SOLUTION (WORKING)

Hydrochloric acid (stock) ..... 50.0 ml

II. Incinerated Specimen Staining

1. Dip incinérated specimen in a thin celloidin solution (2 gm Parloidion to 50 ml absolute alcohol and 50 ml ethyl ether in tightly stoppered bottle. Take 15 ml of this stock solution and make up to 2000 ml with equal parts absolute alcohol and ethyl ether).
2. Air dry slides for a few minutes, then rapidly hydrate to distilled water.
3. Stain with the Prussian blue reaction for 15 minutes.
4. Rinse in distilled water, several. changes.
5. Air dry or dry in 60 ° C oven.
6. Mount with Permount or Histoclad.

RESULTS

Iron oxide - blue

111. Direct Observation of Prussian blue Reaction on Ash

1. Build a well of paraffin around the specimen.
2. Pour hydrochloric acid-potassium ferrocyanide solution in this well and observe under the microscope.

RESULTS

Iron oxide blue

REFERENCE. Fenton, R. H., Johnson, F. B. and Zimmerman, L. E.: *J. Histochem. Cytochem. 12:153 –155, 1964.* Copyright by Williams and Wilkins Co.

LIMES METHOD FOR FERRIC AND FERROUS IRON

FIXATION. 10% buffered neutral. formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

POTASSIUM FERROCYANIDE SOLUTION

Potassium ferrocyanide ($K_4Fe(cn)_6 3H20$) ..... 0.4 gm
0.06 *N* Hydrochloric acid (1 ml concentrated HC1 + 199 ml distilled water) ..... *40.0* ml

POTASSIUM FERRICYANIDE SOLUTION

0.6 *N* Hydrochlorie acid (see above) ..... *40.0 ml*

Make solutions fresh before use.

BASIC FUCHSIN SOLUTION

Basic fuchsin ..... 0.5 gm
Distilled water ..... 100.0 ml
Glacial acetic acid ..... 1.0 ml

STAINING PROCEDURE. Use control slide. Use chemically clean glassware.

1. Deparaffinize and hydrate to distilled water. r
2. For Ferric iron place section in potassium ferrocyanide solution for 1 hour. For Ferrous iron place section in potassium ferricyanide solution for 1 hour.

3. Wash well in 1% aqueous glacial acetic acid.

each.

7. Mount with Permount or Histoclad.

RESULTS

erric iron -dark prussian blue

errous iron -dark turnbull's blue

ackground - light red

Vote: For criticai work omit counterstain.

REFERENCE. Lillie, R. D.: *Histopathologic Technic and Practical Histochemis-*

Nuclei -red

Cytoplasm light pink

REFERENCE. Mallory, F. B., and Wright, J. *H.: Pathological Technique,* 8th ed., Philadelphia, W. B. Saunders Co., *1924, p. 207.*

PERLS' METHOD FOR IRON

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut, paraffin sections at 6 microns.

SOLUTIONS

10% POTASSIUM FERROCYANIDE SOLUTION (STOCK)

(See page *179)*

10% HYDROCHLORIC ACID SOLUTION (STOCK)

Hydrochloric acid, concentrated ..... 10.0 ml

Distilled water ..... 90.0 ml

POTASSIUM FERROCYANIDE-HYDROCHLORIC ACID SOLUTION (WORKING)

Potassium ferrocyanide solution, (stock) ..... *70.0* ml

Hydrochloric acid solution, (stock), ... 30.0 ml

REFERENCE. Perls, *M.: Virchow. Arch. Path. Anat. 39.42,* 1867.

SCHMORL'S METHOD FOR REDUCING SUBSTANCES

FIXATION. 10% buffered neutral. formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% FERRIC CHLORIDE SOLUTION (STOCK)

Ferric chloride ..... 1.0 gm

Distilled water .....100.0 ml

0.1% POTASSIUM FERRICYANIDE SOLUTION (STOCK)

Potassium ferricyanide ..... 0.1 gm

Distilled water .....100.0 ml

FERRIC CHLORIDE-POTASSIUM FERRICYANIDE SOLUTION (WORKING)

Ferric chloride (stock) .....150.0 ml

REFERENCE. Barka, T. and Anderson, P.: *Histochemistry Theory, Practice, and Bibliography,* New York, Harper & Row Publishers, Inc., Hoeber Med. Div., 1963, p. 185 -186.

AFIP METHOD FOR LIPOFUSCIN

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

KINYOUN'S CARBOL FUCHSIN SOLUTION

(See page 218)

1% ACID ALCOHOL SOLUTION

(See page 38)

PICRIC ACID SOLUTION

Picric acid, approximately ..... 1.18 gm

Distilled water ..... 100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Kinyoun's carbol fuchsin solution for 1 hour.
3. Rinse in distilled water.

SOLUTIONS

011 RED O IN PROPYLENE GLYCOL SOLUTION

(See page 141)

85% PROPYLENE GLYCOL SOLUTION

(See page 141)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.

2. ORO in propylene glycol solution for 72 hours.

3. 85% propylene glycol solution for 1 minute. Agitate slide.

4. Distilled water, two changes for 2 minutes each.
5. Mayer's hematoxylin solution for 5 minutes.

3. Prepare developing solution and pour this over slides until the urates turn black and the connective tissues are yellow.
4. Wash qiúckly'in hot water (58 ° C).
5. Dehydrate in absolute alcohol, and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Urates - black
Background - yellow

Note. If specimen contains bone, decalcify in absolute alcohol to which 2% nitric acid has been added. Change solution every 24 hours until bone is softened. Wash in absolute alcohol for 24 hours.

REFERENCE. De Galantha, *E.*: ***Amer.*** *J. Clin. Path.* 5:165 -166, 1935. Copyright by Williams and Wilkins Co.

Chapter 12

Methods For Nerve Cells And Fibers

RAMON Y CAJAL'S METHOD FOR ASTROCYTES

FIXATION. Formalin ammonium bromide for 1 day at 37 °C or 10% buffered neutral formalin (see *Note).*

ECHNIQUE. Cut frozen sections at 15 to 30 microns.

SOLUTIONS

FORMALIN AMMONIUM BROMIDE SOLUTION

Formalin, 37 — 40% (Merck's blue label) ..... 15.0 ml

Ammonium bromide ..... , 2.0 gm

5. Rinse well in several changes of distilled water.

each.

8. Mount with Permount or

Histoclad. RESULTS

\strocytes and their processes -black

3ackground -unstained or light brownish purple

Nerve cells - pala red

2.5% SODIUM HYDROXIDE (STOCK)

c

AMMONIACAL SILVER SOLUTION (WORKING)

Silver nitrate solution (stock) ..... 20.0 mi.

Alcohol, 100% ..... 10.0 ml

Ammonium hydroxide 28% ..... 1.8 mi.

Sodium hydroxide (stock) ..... 1.5 ml

This solution should be placed finto a warmed dish on a hot plate and covered.

REDUCING SOLUTION

SOLUTIONS

15% ALCOHOL SOLUTION

Alcohol, 100% ..... 15.0 ml

Distilled water ..... 85.0 ml

0.05% POTASSIUM PERMANGANATE SOLUTION

Potassium permanganate ..... 0.05 gm

Distilled water ..... 100.0 ml

0.5% PHOSPHOMOLYBDIC ACID SOLUTION

Phosphomolybdic acid ..... *0.5* gm

9. Rinse in distilled water.

10. Ammoniacal silves nitrate solution for 1 minute.

11. Reducing solution. Sections will float out evenly on the surface of this solution and a brown color will appear within 1 minute.

12. Rinse in distilled water.

13. Sodium thiosulfate solution for 1 minute.

14. Rinse in distilled water, three changes.

15. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, three changes each.

16. Mount with Permount or Histoclad.

RESULTS. Degenerating axons stain with silver as fragmented and swollen fibers.

REMARKS. Guillery et al modified this method for use with paraffin embedded sections, see page 190.

Note. This step is highly criticai: too long treatment will inhibit subsequent silver impregnation of ali fibers, too short treatment will cause impregnation of too many normal fibers. The optimum duration varies froco specimen to specimen and can best be established in a trial run, treating three sections for 5, 7, and 9 minutes respectix,cly. During treatment sections must be turned oves froco time to time in order to insure an even effect.

REFERENCE. Nauta, W. J. H., and Gygax, P. A.: *Stain Techn. 29:91-93,* 1954— Copyright by Williams and Wilkins Co.

BIELSCHOWSKY'S METHOD FOR AXIS CYLINDERS AND DENDRITES

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

2% SILVER NITRATE SOLUTION

Silver nitrate ..... 2.0 gm
Distilled water ..... 100.0 ml

10% SILVER NITRATE SOLUTION

Silver nitrate ..... 10.0 gm
Distilled water ..... 100.0 ml

40% SODIUM HYDROXIDE SOLUTION

Sodium hydroxide ..... 40.0 gm
Distilled water ..... 100.0 ml

AMMONIACAL SILVER SOLUTION

Silver nitrate, 10% ..... 5.0 ml

Dissolve the resulting precipitate by adding concentrated ammonium hydroxide drop by drop until the precipitate is just dissolved. Filter and dilute to 20 ml with distilled water. Make fresh.

20% FORMALIN SOLUTION

Formalin, 37 - 40% ..... 20.0 ml

Tap water .....80.0 ml

GOLD CHLORIDE SOLUTION

Gold chloride, 11^{9-10} aqueous .....3 drops

Distilled water .....10.0 ml

BODIANS METHOD FOR NERVE FIBERS AND NERVE ENDINGS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% PROTARGOL SOLUTION

Protargol ..... 1.0 gm

Distilled water ..... 100.0 ml

Sprinkle the protargol on the surface of the water and allow it to remain undisturbed until it dissolves.

REDUCING SOLUTION

Hydroquinone 1 0 g m

Formalin, 37 — 40% ..... 5.0 ml

c

2. Place slides in protargol solution and add 4 to 6 gm of clean copper shot per 100 ml of solution. Let stand at 37 °C for 48 hours.

3. Rinse in distilled water, three changes.

4. Reducing solution for 10 minutes.

5. Rinse in distilled water, three changes.

6. Tone in gold clãoride solution for 10 minutes.

7. Rinse in distilled water, three changes.

8. Develop in oxalic acid solution under the microscope until background is gray and nerve fibers appear clearly, approximately 3 - 5 minutes.

9. Rinse in distilled water, three changes.

10. Sodium thiosulfate for 5 minutes.

11. Rinse in distilled water.

12. Counterstain, if desired, with aniline blue solution (two or three quick dips, to give a light blue background) or lissamine fast red solution (see Technique for Counter-staining with Lissamine Fast Red Solution below).

13. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

14. Mount with Permount or Histoclad.

RESULTS.(If aniline blue solution is used)

or

erve fibers - black

ackground - blue

SOLUTIONS

20% SILVER NITRATE SOLUTION

(See page 187)

BORIC ACID BUFFER SOLUTION

Boric acid ..... 12.4 gm

Distilled water ..... 1000.0 ml

BORAX BUFFER SOLUTION

Sodium borato, tetra (Borax) ..... 19.0 gm

Distilled water ..... 1000.0 ml

1% SILVER NITRATE SOLUTION

Silver nitrate ..... 1.0 gm

Distilled water ..... 100.0 ml

10% PYRIDINE SOLUTION

5. Remove slides, drain excess solution and placa in reducing solution for at least *2* minutes.
6. Wash in running water for *3* minutes, then rinse in distilled water.
7. Tone in gold chloride solution for *3* minutes.
8. Rinse briefly in distilled water.
9. Oxalic acid solution for *3* - 5 minutes. Examine microscopically until axons are thoroughly blue-black.
10. Rinse in distilled water.
11. Sodium thiosulfate solution for 5 minutes then wash in running water for 5 minutes.
12. Dehydrate in 95% alcohol, absoluta alcohol, and clear in xylene, two changes each.
13. Mount with Permount or Histoclad.

RESULTS

Axis cylinders - blue to black
Nerves and verve endings - black

ackground -gray to rose

EFERENCE. Holmes, *W.: Anat. Rec. 86:157-187, 1943.*

HIRANO-ZIMMERMAN METHOD FOR NERVE CELLS AND FIBERS

FIXATION. 10% buffered neutral formalin. TECHNIQUE.
Cut parffin sections at *12 -15* microns.

SOLUTIONS

10% SILVER NITRATE SOLUTION

Silver nitrate ..... 10.0 gm

3. Place sections directly into ammonia water solution for 3 minutes.
4. Place sections directly in formalin-tap water solution for 3 minutes. Tissue becomes black-brown.
5. Rinse in distilled water, two changes.
6. Place sections again into silvar nitrate solution for 3 to 5 minutes.
7. Repeat steps 3 through 5. Tissue becomes darker in color.

each

12. Mount with Permount or Histoclad.

RESULTS

Nucleolus and nuclear membrana - black
Neurofibrils, dendrites and axis cylinders - black
Cytoplasm of astrocytes and the cytoplasmic membranas of

nacrophages - gray
Senile plaques - black
Various lipid granules are unstained, but each granule is clearly outlined.

REFERENCE. Hirano, A., and Zimmerman, *H. M.: Arch. Neurol. 6:114* -122, 1962.

PTAH METHOD FOR CENTRAL NERVOUS SYSTEM (C.N.S) TISSUE

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% POTASSIUM PERMANGANATE SOLUTION
(See page 102)

5% OXALIC ACID SOLUTION
(See page 79)

PHOSPHOTUNGSTIC ACID HEMATOXYLIN (PTAH) SOLUTION

3. Wash in running water for 15 minutes.
4. Lugol's iodine solution (soe pago *42)* for *15* minutes.
5. Decolorize in *95%* alcohol for one hour or longer. *DO NOT USE HYPO*.*
6. Distilled water for 5 minutes.

7. Oxidize in potassium permanganate solution for *5* minutes.

8. Decolorize in oxalic acid solution for *5* minutes.

.9. Rinse well in distilled water followed by running water for *5 -10* minutes.

10. PTAH solution for *24* hours.
11. 95% alcohol for one quick dip.

e l -

*Sodium thiosulfate should not be used to decolorize sections after treatment with iodine because it is likely to impair subsequent staining.

DIFFERENTIATING SOLUTION

Aniline ..... 120.0 ml

Chloroform ..... 180.0 ml
Ammonium hydroxide, 28% ..... 20 drops

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water. Carry slides through one at a time.
2. Phosphomolybdic-alcohol solution for 3 minutes. Drain.
3. Absolute alcohol-chloroform solution until section becomes translucent.
4. Place slide on staining rack and flood section with crystal violet solution for 30 seconds. Blot dry.
5. Flood section with potassium bromide solution for one minute. Blot dry.
6. Differentiating solution for 30 seconds. If overdifferentiated, restain.
7. Rinse in xylene, several changes. Check with microscope after first xylene.
8. Mount with Permount or Histoclad.

RESULTS

Glial fibers - deep violet

1% FORMALIN SOLUTION

(See page 90)

GOLD CHLORIDE SOLUTION

Gold chloride 10 gm

Distilled water ..... 500.0 ml

5% SODIUM THIOSULFATE

(See page 89)

HORTEGAS CARBOL-MENE-CREOSOTE MIXTURE

Creosote ..... 10.0 ml

Phenol crystals, melted ..... 10.0 ml

U

SOLUTIONS

BOHMER'S HEMATOXYLIN SOLUTION

(See page 34)

1% THIOFLAVIN TFS SOLUTION

Thioflavin, TFS[1]	1.0 gm
Distilled water	100.0 ml

1% GLACIAL ACETIC ACID SOLUTION

(See page 94)

APATHY'S MOUNTING MEDIA

(See page 156)

STAINING PROCEDURE. Mount frozen sections on glass slide and allow sections to dry for 2 hours.

1. Bohmer's hematoxylin solution for 2 minutes.
2. Wash thoroughly in running water.
3. Thioflavin TFS solution for 3 minutes.
4. Rinse in distilled water.
5. Differentiate in glacial acetic acid solution for 6 minutes.
6. Wash in tap water, then rinse in distilled water.
7. Mount with Apathy's mounting media.

Examine sections with fluorescent microscope.

RESULTS

Myelin figures -bluish white fluorescente

REFERENCE. Casper, J., and Wolman, M.: *Lab. Invest.* 13:27 -31, 1964. Copyright by International Academy of Pathologists.

KLÜVER - BARRERA METHOD^"FOR MYELIN AND NERVE CELLS

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 15 microns.

SOLUTIONS

0.1% LUXOL FAST BLUE SOLUTION

Luxol fast blue, MBSt	0.1 gm
Alcohol, 95%	100.0 ml

Dissolve dye in alcohol. Add 0.5 ml of 10% glacial acetic acid to each 100 ml. Solution is stable.

*Scheuler & Co., Ltd., New York, N. Y .

tE. I. Du Pont de Nemours and Co., Wilmington, Dei.

0.1% CRESYL ECHT VIOLEI SOLUTION

Cresyl echt violet ..... 0.1 gm

Distilled water ..... 100.0 ml

just before using, add 15 drops of 10% glacial acetic acid. Filter.

0.05% LITHIUM CARBONATE SOLUTION

Lithium carbonate ..... 0.05 gm

Distilled water ..... 100.0 ml

70% ALCOHOL SOLUTION

Alcohol, 100% ..... 70.0 ml

Distilled water ..... 30.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to 95% alcohol.

SOLUTIONS

GALLOCYAN I N SOLUTION

Chromium potassium sulfate (chrom alum) ..... 10.0 gm

Distilled water ..... 200.0 ml
Gallocyanin ..... 0.3 gm

Heat slowly to boiling, boil for 12-25 minutes with frequent stirring. Cool slowly to room temperatura. Filter bafore using.

0.5% PHLOXINE B AQUEOUS SOLUTION

(See page 106)

5% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 86)

1% GLACIAL ACETIC ACID SOLUTION

(See page 94)

0.05% FAST GREEN FCF SOLUTION

Fast green FCF 0.05

op

Note. The, phloxine is differentiated at step 9. All tissue structures are decolorized at this time except myelin and erythrocytes. Over-differentiation does not occur within limite.

REFERENCE. Lapham, L. W., Johnstone, M. A., and Brundjar, K. H.: *J. Neuropath. Exp. Neurol.* 23:156-160, 1964.

WOELCKE'S METHOD FOR MYELIN SHEATH

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 15 microns.

SOLUTIONS

2.5% FERRIC AMMONIUM SULFATE SOLUTION

(See page 140)

10% ALCOHOLIC HEMATOXYLIN SOLUTION (STOCK)

Hematoxylin ..... 10.0 gm
Alcohol, 100% ..... 100.0 ml
Solution should be ripened for 6 months.

SATURATED LITHIUM CARBONATE SOLUTION

(S e e p a g e 3 8)

HEMATOXYLIN SOLUTION (WORKING)

Mix the following ingrediente in order listed just before use:

0^

RESULTS

yelin sheath - blue

ackground - clear
Glial cells - black
Nucleoli of neurons - black

REFERENCE. Personal communication with A. Pentschev, M.D.: Armed Forces Instituto of Pathology, Washington, D.C. 20305

BOSCHS METHOD FOR NEGRI BODIES

FIXATION. Bouin's (soe *Note)* or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(Soe pape 33)

PHENOL-PHLOXINE B SOLUTION

Phloxine B ..... 1.5 gm

Distilled water ..... 5.0 ml

ix and add, with vigorous shaking:

Phenol, 5%, to make ..... 100.0 ml

Add, drop by drop, with vigorous shaking, 20 — 30% glacial acetic acid until solution becomes cloudy. Placo in a closed vessel and heat in a 50 - 56 °C oven for 12 - 24 hours. Use the supernatant as the staining solution.

10. Dehydrate in absolute alcohol and clear in xylene, two changes each.

11. Mount with Permount or Histoclad.

RESULTS

egri bodies -deep red

uclei, nucleoli and nissl granules - deep blue

ackground - light pink

7. Stain 8 minutes in eosin solution. Wash thoroughly in water and dehydrate by short dips in 50% alcohol, 70% alcohol, 80% alcohol, and 90% alcohol and longer dips in 95% alcohol. Use two or three changes of absoluta alcohol, 4 minutes each.

S. Blot with filter papar.

9. Clear in two changes of xylene, 4 minutes each.

10. Mount with Permount or Histoclad.

RESULTS

Negri bodies - deep red

Nuclei - light blue

Note. To obtain a good stain it is important not to overstain in Harris' hematoxylin, to differentiate very well in diluta HC1 and after eosin, to follow exactly the instructions regarding the dips.

4. Wash quickly in tap water.
5. Differentiate each slide individually, by gently agitating in *90%* alcohol until sections are faint violet color.
6. Dehydrate in *95%* alcohol, absoluta alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Negri bodies - deep magenta

Cytoplasm - bluish violet

rythrocytes -copper

REMARKS. This technic is excellent for demonstrating Negri bodies in Zenker-fixed specimens only. Attempts to post-Zenkerize sections prior to staining so far have proveu useless.

Note. The important consideration at this point is to achieve a slightly alkaline solution. It may be necessary to vary the amount of potassium hydroxide to accomplish this.

REFERENCE. Schleifstein, J.: ***Amer.*** *J. Public Health 27:1283 -1285, 1937.*

ALDEHYDE-THIONIN-PAS METHOD FOR CENTRAL NERVOUS SYSTEM

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Ctit paraffin sections at 6 microns.

SOLUTIONS

0.5% SULFURIC ACID SOLUTION

Sulfuric acid, concentrated ..... 0.5 ml

0.5% PERIODIC ACID SOLUTION

(See page 72)

SCHIFF REAGENT SOLUTION

(See page *159)*

1% ORANGE G SOLUTION

(See page 114)

1% GLACIAL ACETIC ACID SOLUTION

(See page *94)*

5% PHOSPHOTUNGSTIC ACID SOLUTION

(See page 86)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Equal parts of sulfuric acid solution and potassium permanganate solution for 2 minutes.
3. Bleach with potassium metabisulfite for 1 minute.
4. Rinse well in distilled water.
5. Aldehyde thionin solution in an air stoppered container for approximately *50* minutes.
6. Rinse in distilled water.
7. Periodic acid solution for 5 minutes.
8. Rinse in distilled water.
9. Schiff's reagent solution for 15 minutes.
10. Wash in running water for *15* minutes.
11. Orange G solution for 3 minutes.
12. Phosphotungstic acid solution for 1 minute.
13. Rinse briefly in glacial acetic acid solution.
14. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes each.
15. Mount with Permount or Histoclad.

RESULTS

Nissl substances	blue-black
PAS positive materiais	red
Background	-various shades depending on amount of differentiation

REMARKS. This technic has been used to demonstrate alpha, beta, gamma, and delta cells of the pituitary and has been modified slightly to demonstrate Nissl substances. *Note.* Schiff reagent should be checked and renewed frequently.

REFERENCE. Author unknown.

EINARSON'S METHOD FOR NISSL SUBSTANCE

FIXATION. m[a]io buffered neutral formalin, Zenker's, or Zenker-formol.

TECHNIQUE.

Cut paraffin sections at 6 microns.

SOLUTIONS

GALLOCYANIN SOLUTION

Gallocyanin, (Hartman, Leddon) ..... 0.15 gm

Chromium potassium sulfate ..... 5.0 gm

Distilled water ..... 100.0 MI.

Dissolve the chrome alum in warm water, add gallocyanin, and boil gently for 10 minutes. Cool and filter. This solution keeps well for about one week.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Placa sections in gallocyanin solution for 1 hour at 56 °C, or leave overnight at room temperatura.
3. Rinse in water.
4. Dehydrate in 95% alcohol, absoluta alcohol, and clear in xylene, two changes each.
5. Mount with Permount or Histoclad.

RESULTS

issl substance - blue

REFERENCE. Einarson, L.: ***Amer.*** *J. Path.* 8:295 -309, 1932.

VOGT'S METHOD FOR NERVE CELL PRODUCTS'

FIXATION. 10% buffered neutral formalin.

STAINING PROCEDURE

1. Deparaffinize and carry through absolute alcohol, two changes.

2. Let stand in absolute alcohol for 2 hours.

3. Working cresyl echt violet solution for 40 - 60 minutes.

4. Differentiate rapidly in 95% alcohol, then dehydrate in absolute alcohol, and clear in xylene, two changes each.

5. Mount with Permount or Histoclad.

RESULTS

SUBSTRATE SOLUTION (WORKING)

Copper sulfate solution (stock) ..... 0.2 ml

Aminoacetic'acid solution (stock) ..... 0.2 ml
Acetato buffer solution (stock) ..... 5.0 ml
Supernatant solution ..... 0.8 ml

5% AMMONIUM SULPHIDE SOLUTION

Ammonium sulphide, yellow .....5.0 ml

Distilled water .....100.0 ml

10% SILVER NITRATE SOLUTION

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(See page 198)

S. Wash in 10% formalin until the white precipitate no longer fornis, about six changes.

9. Ammoniacal silver solution until impregnation of the terminal portion of the nerve fibers has oceurred. May take several hours.

10. Ammonia water solution for 2 minutes.

11. Glacial acetic acid solution for 2 minutes.

12. Distilled water for 2 minutes.

13. Sodium thiosulfate solution for 5 minutes.

14 Distilled water for 30 minutes.

15. Dehydrate in 95% alcohol, absolute alcohol, and olear in xylene, two changes each.

16. Mount with Permount or Histoclad.

RESULTS

Motor end plates and associated nerve fibers - black

Background - pile grey

REFERENCE. Gwyn, D. G., and Heardman, *V.: Stain Techn.* 40:15 -18, 1965. Copyright by Williams and Wilkins Co.

SEVIER-MUNGER METHOD FOR NEURAL TISSUES

FIXATION. 10% buffered neutral. formalin. Avoid chromate fixatives.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

20% SILVER NITRATE SOLUTION

(See page 187)

10% SILVER NITRATE SOLUTION

To 50 ml of 10% silver nitrate solution add ammonium hydroxide (28-30%) drop by drop until the dark brown precipitate which forros has almost

vigorously between drops. Avoid complete decolorization. The end point is a slightly cloudy solution. Add to this solution *0.5* ml of sodium carbonate solution. Shake well. Add *25* drops of ammonium hydroxide. Shake well. Solution should now be crystal clear. Filter finto a *125* ml Erlenmeyer flask. Cover.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Preheat 20% silver nitrate solution to 60'C for 15 minutes. Add slides to warm silver solution and let them remain in the oven for 15 minutes.
3. Rinse one slide at a time in distilled water, then place in a clean coplin jar.
4. To working ammoniacal silver solution add 10 drops of the formalin solution, shaking gently while adding formalin. Pour quickly oven slides, let develop for 5 to *30* minutes until golden brown. Keep in motion to avoid precipitate.
5. Examine microscopically. *Do not wash.* Developing should be neither too fast nor too slow.
6. Rinse well in fresh tap water, three changes.
7. Sodium thiosulfate solution for 2 minutes.
8. Wash in water.
9. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes each.
10. Mount with Permount or Histoclad.

RESULTS

Large and small peripheral neurities	- black
Axons	- black
Myelin sheath	- light brown
Collagen and muscle	- brown
Argentaffin granules	- black

Note. Sections of the central nervous system have also been stained with this technic. The cerebellar basket cell and axons of the Purkinje cell became beautifully impregnated. Within the brain stem and spinal cord, large fibers are stained but small fibers are not visualized. The optic and olfactory nerves do not stain

REFERENCE. Sevier, A. C., and Munger, B. L.: *J. Neuropath. Exp. Neurol. 24: 130-135,1965.*

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vigorously between drops. Avoid complete decolorization. The end point is a slightly cloudy solution. Add to this solution *0.5* ml of sodium carbonate solution. Shake well. Add *25* drops of affimonium hydroxide. Shake well. Solution should now be crystal clear. Filter finto a *125* ml Erlenmeyer flask. Cover.

STAINING PROCEDURE

3. Deparaffinize and hydrate to distilled water.

4. Preheat *20%* silver nitrate solution to *60'C* for 15 minutes. Add slides to warm silver solution and let them remain in the oven for *15* minutes.

5. Rinse one slide at a time in distilled water, then place in a clean coplin jar.

6. To working ammoniacal silver solution add 10 drops of the formalin solution, shaking gently while adding formalin. Pour quickly over slides, let develop for *5* to 30 minutes until golden brown. Keep in motion to avoid precipitate.

7. Examine microscopically. *Do not wash.* Developing should be neither too fast nor too slow.

8. Rinse well in fresh tap water, three changes.

9. Sodium thiosulfate solution for *2* minutes.

10. Wash in water.

11. Dehydrate in *95%* alcohol, absolute alcohol, and clear in xylene, two changes each.

13. Mount with Permount or Histoclad

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RESULTS

Large and small peripheral neurities	black
Axons	black
Myelin sheath	light brown
Collagen and muscle	brown
Argentaffin granules	black

Note. Sections of the central nervous system have also been stained with this technic. The cerebellar basket cell and axons of the Purkinje cell became beautifully impregnated. Within the brain stem and spinal cord, large fibers are stained but small fibers are not visualized. The optic and olfactory nerves do not stain

REFERENCE. Sevier, A. C., and Munger, B. L.: *J. Neuropath. Exp. Neurol. 24: 130-135,1965.*

RESULTS

Acid fast bacilli	-red
Nocardia filaments	- red
Lepra bacilli	- red
Background	- pale blue

REMARKS. This technic is used in our laboratories for the demonstration of certain strains of Nocardia and lepra bacilli. We find this technic to be the most useful of the acid fast procedures for the demonstration of these pathologic entities for the following reasons: (1) The protection, afforded by the combination of peanut oil and xylene, coats the micro-organisms, (2) the oil coating, applied around the filaments and lepra bacilli, is not dissolved due to the elimination of alcohol froco both the hydration and dehydration process, and (3) the decolorization of the background with a weak solution of aqueous sulfuric acid instead of the usual 1% hydrochloric acid-alcohol does not differentiate too rapidly.

REFERENCE. Fite, C. L., Cambre, P. J., and Turner, M. H.: *Arch. Path. 43:624 -625, 1947.*

KINYOUN'S METHOD FOR ACID FAST BACTERIA

FIXATION. 10% buffered neutral. formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

KINYOUN'S CARBOL FUCHSIN SOLUTION

Basic fuchsin	4.0 gm
Phenol crystals, melted	8.0 ml
Alcohol, *95%*	20.0 ml
Distilled water	100.0 ml

1% ACID ALCOHOL SOLUTION

(See page 38)

METHYLENE BLUE SOLUTION (STOCK)

Methylene blue	1.4 gm
Alcohol, *95 /*[cl]o	100.0 ml

METHYLENE BLUE SOLUTION (WORKING)

Methylene blue solution (stock)	10.0 ml
Tap water	90.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Kinyoun's carbol fuchsin solution at 56 'C for 1 hour.
3. Wash well in running water.
4. Differentiate in two changes of acid alcohol solution until tissue is pale pink.
5. Wash well in running water.

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6. Counterstain in working methylene blue solution for 2 seconds.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Acid-fast bacteria -bright red
Background - light blue

REFERENCE. Kinyoun, J. J.: *Amer. J. Public Health* 5:867, 1915. (AFIP modification)

TRUANTS FLUORESCENT METHOD FOR ACID FAST ORGANISMS

FIXATION. 10% buffered neutral formalin.

TEC H N1Q U E. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

AURAMINE-RHODAMINE SOLUTION

Auramine	1.5 gm
Rhodamine B	0.75 gm
Glycerin, U.S.P.	75.0 ml
Phenol crystals, (melted)	10.01111
Distilled water	50.0 ml

1% ACID ALCOHOL SOLUTION

(See page 38)

STAINING PROCEDO RE. Use control slide.

3. Deparaffinize and hydrate to distilled water.
4. Weigert's irou hematoxylin solution for 10 minutes.
5. Wash in running water 10 minutes.
6. Auramine-Rhodamine solution at 60 °C for 10 minutes.
7. Wash in tap water.
8. Differentiate in acid alcohol solution to remove excess stain.
9. Wash in running water.
10. Rinse **in** distilled water.
11. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
12. Mount with Permount or Histoclad.

RESULTS

Bacilli will fluoresce reddish yellow.

Artifacts will not have the reddish color and will remain yellow,.

REMARKS. The Weigert's hematoxylin used as a fluorescence inhibitor, also gives a good cell stain. The use of thinner sections will reduce the background staining. **REFERENCE.** Truant, J. P.: *Henry Ford Hospital Med. Bull.* 10:287, 1962.

ZIEHL - NEELSEN METHOD FOR ACID -FAST BACTERIA

FIXATION. Any well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

CARBOL FUCHSIN SOLUTION

Phenol crystals, (melted) ..... 2.5 ml
Alcohol, 100% 5.0 ml
Basic fuchsin .....0.5 gm
Distilled water ..... 50.0 ml
Filter before use.

1% ACID ALCOHOL SOLUTION

(See page 38)

1% SULFURIC ACID SOLUTION

(See page 102)

METHYLENE BLUE SOLUTIONS

(See page 218)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Carbol fuchsin solution for 30 minutes.
3. Wash well in running water.
4. Decolorize with acid alcohol solution or sulfuric acid solution until sections are pale pink.
5. Wash thoroughly in running water for 8 minutes.
6. Counterstain by dipping one slide at a time in working methylene blue solution. Sections should be pale blue.
7. Wash with tap water, then rinse in distilled water.

S. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

9. Mount with Permount or Histoclad.

RESULTS

Acid-fast bacilli - bright red
Erythrocytes - yellowish orange
Other tissue elements - pale blue

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p.275. (AFIP modification)

WADE'S METHOD FOR ACID FAST ORGANISMS

FIXATION. Zenker's or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

DEPARAFFINIZING SOLUTION

Turpentine, rectified ..... 2 parts
Paraffin oil (liquid petrolatum), heavy ..... 1 part

CARBOL - NEW FUCHSIN SOLUTION

New fuchsin, Magenta 111 ..... 0.5 gm
Phenol crystals, melted ..... 5.0 ml
Alcohol, 100% ..... 10.0 ml
Distilled water to make ..... 100.0 ml

5% SULFURIC ACID SOLUTION

Sulfuric acid, concentrated ..... 5.0 ml
Distilled water ..... 95.0 ml

1% POTASSIUM PERMANGANATE SOLUTION

(See page 102)

2% OXALIC ACID SOLUTION

(See page 195)

MODIFIED VAN GIESON SOLUTION

Acid fuchsin ..... 0.01 gm
Picric acid ..... 1.0 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE. Use control slide.

3. Deparaffinizing solution, two changes, 5 minutes each.
4. Drain, wipe off excess fluid, blot to opacity, and place in water.
5. Carbol-new fuchsin solution, overnight.
6. Wash in water for 2 minutes.
7. Full strength formalin for 5 minutes (see *Note).* The bacilli become blue. The sections may turn blue or remain predominantly reddish.
8. Wash in running water for 2 minutes.
9. Sulfuric acid solution for 1 minute, then wash well in running water for 5 minutes.
10. Potassium permanganate solution 3 minutes. Rinse in tap water.
11. Oxalic acid solution to bleach for 30 - 60 seconds, then wash thoroughly in running water. Use 5% oxalic acid for specimens that do not bleach readily.
12. Modified van Gieson solution for 3 minutes.
13. Dehydrate rapidly in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

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RESULTS

Acid-fast bacilli	-deep ultramarino blue, or blue-black
Connective tissue elements	-red
Background	- yellowish

Note. The quality of the formalin is criticai. Fite specified particularly freshly redistilled formalin, but the reagent grade is much more convenient and thoroughly dependable. The blue color of the bacilli is permanent. If it is suspected that there is not total demonstration of bacilli by this method, step 1 may be prolonged for 4 - 6 hours for "restoration" of poorly staining bacilli.

REFERENCE. Wade, *H. W.: Amer. J. Path. 28:157, 1952.*

BROWN AND BRENN METHOD FOR GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% CRYSTAL VIOLEI SOLUTION

Crystal violet 10 gm
Distilled water ..... 100.0 ml

5% SODIUM BICARBONATE SOLUTION

Sodium bicarbonato ..... 5.0 gm
Distilled water ..... 100.0 ml

GRAM'S IODINE SOLUTION

(Soe pago 41)

ETHYL ETHER - ACETONE SOLUTION

Ethy1 ether ..... 50.0 ml
Acetone ..... 50.0 ml

0.25% BASIC FUCHSIN SOLUTION (STOCK)

Basic fuchsin ..... *0.25* gm
Distilled water ..... 100.0 ml

BASIC FUCHSIN SOLUTION (WORKING)

Basic fuchsin solution (stock) ..... 10.0 ml
Distilled water ..... 100.0 ml

PICRIC ACID-ACETONE SOLUTION

Picric acid	0_1 gm
Acetone	100.0 ml

ACETONE-XYLENE SOLUTION

Acetone	50.0 ml
Xylene	50.0 ml

STAINING PROCEDURE. Use control slide.

3. Deparaffinize and hydrate to distilled water.
4. Placo slides on a staining rack. Pour on approximately 1 ml (or 20 drops) of crystal violet solution and add 5 drops of sodium bicarbonato solution for 1 minute. Agitate gently. (If preferred, the two solutions may be mixed just before use).
5. Rinse in tap water.
6. Flood slides with Gram's iodine solution for 1 minute.
7. Rinse in water and blot with filter paper to complete dryness.
8. Decolorize with ethyl ether-acetone solution dropped on slides until no more color runs off.
9. Working basic fuchsin solution for 1 minute. Wash in water. Blot gently, but do not allow sections to dry completely.
10. Dip in acetone to start differentiation reaction.
11. Differentiate immediately with picric acid-acetone solution until sections are yellowish pink.
12. Rinse quickly in acetone, then in acetone-xylene solution.
13. Clear in xylene, several changes.
14. Mount with Permount or Histoclad.

RESULTS

Gram positive bacteria	- blue
Gram negative bacteria	- red
Filaments of Nocardia and Actinomyces	- blue
Nuclei	- red
Other tissue elements	- yellow

REMARKS. This method is invaluable in the demonstration of the filaments of Nocardia and Actinomyces. It must be realized, however, that these filaments are not completely or strongly Gram positive. It is possible to obtain either Gram positive or Gram negative results depending on the degree of differentiation. It is suggested therefore, that at least two slides be run with varying degrees of differentiation in order to lessen the possibility of Quer-differentiation at step 8 of the staining procedure. For the study of Gram positive and Gram negative bacteria, R. D. Taylor's modification of Brown and Brenn technic on pago 226 is recommended. We find this to be the best technic for demonstrating micro-organisms. This does not imply that this technic (B & B) is not useful for demonstrating Gram positive and, Gram negative bacteria.

REFERENCE. Brown, J. H., and Brenn, *L.: Bull. Johns Hopkins Hosp.* 48:69 -73, 1931. (AFIP modification)

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BROWN - HOPPS METHOD FOR GRAM POSITIVE AND . GRAM NEGATIVE BACTERIA

FIXATION. 10% buffered neutral formalin

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% CRYSTAL VIOLEI SOLUTION

(See page 222)

GRAMS IODINE SOLUTION

(See page 41)

CELLOSOLVE

(Ethylene glycol monoethyl ether).

0.5% BASIC FUCHSIN SOLUTION

Basic fuchsin ..... 0.5 gm
Distilled water ..... 100.0 ml

GALLEGO'S DIFFERENTIATING SOLUTION

Distilled water..... 50.0 ml
Formalin, 37 — 40% ..... 1.0 ml
Glacial acetic acid ..... 0.5 ml

1.5% TARTRAZINE SOLUTION

Tartrazine..... 1.5 gm
Distilled water ..... 100.0 ml

STAINING PROCEDURE. Use control slide.

4. Deparaffinize and hydrate to distilled water.
5. Place slides on staining rack and pour on crystal violet solution for 2 minutes.
6. Rinse in distilled water.
7. Mordant in Gram's iodine solution for 5 minutes.
8. Rinse in distilled water.
9. Differentiate in Cellosolve until blue color no longer streams away froco the section (approximately 5 -10 seconds) (see *Note).*
10. Quickly rinse in distilled water (see *Note).*

S. Basic fuchsin solution for 5 minutes.

11. Rinse in distilled water.
12. Gallego's differentiating solution for 5 minutes (differentiates and "fixes" the basic fuchsin).
13. Rinse thoroughly in distilled water.
14. Blot lightly, only to remove excess water (not to dryness)
15. Tartrazine solution for 3 seconds. Immediately blot away excess, but not to dryness (see *Note).*
16. Cellosolve, three changes, for six quick dips in each (see *NQte).*

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17. Xylene, three changes, for ten. dips each. Slides may remain in xylene until ready for mounting.

18. Mount with Permount or Histoclad.

RESULTS

Gram positive bacteria -blue
Gram negativo bacteria red
Background yellow

Notes. Preferably, ali stains and solutions are applied to the slide which is in a horizontal position, except for steps 6, 14, and 15, in which case the slide is dipped finto the solutions contained in coplin jars. The stain works satisfactorily on tissues fixed in 10% formalin, (Na phosphate or Na acetato buffered), glutaraldehyde (2.59%, with phosphate buffer), and formol-Zenker's fluid. It does *not* work well on Bouin's fixed tissues.

Steps 6, 7, 13, and 14 are criticai in regard to timing.

REFERENCE. Brown, R. C., and Hopps, H. C.: Geographic Pathology Division, Armed Forces Instituto of Pathology, Washington, D.C. 20305. (Method to be published in detail at a lates date).

MACCALLUM-GOODPASTURE METHOD FOR GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

FIXATION. Any well-fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

GOODPASTURE'S SOLUTION

Basic fuchsin	0.59	gm
Aniline	1.0	1111
Phenol crystals (melted)	1.0	1111
Alcohol, 30%	100.0	ml

GRAM'S IODINE SOLUTION

(See page 41)

STIRLING'S GENTIAN VIOLEI SOLUTION

Gentian violet (crystal violet)	5.0 gm
Alcohol, 100%	10.0 ml
Aniline	2.0 ml
Distilled water	88.0 ml

SATURATED PICRIC ACID SOLUTION

(See page 39)

STAINING PROCEDURE. Use control slide.

Aw

2. Deparaffinize and hydrate to distilled water.
3. Goodpasture's solution for 10 minutes.

3. Rinse in distilled water.
4. Differentiate in full strength formalin for a few minutes (fixes Goodpasture stain).
5. Wash in running water for 3 minutes.
6. Saturated picric acid solution for 3 to 5 minutes.
7. Rinse in distilled water. S. Differentiate in
95% alcohol for 30 seconds.
12. Rinse in distilled water.
13. Stirling's gentian violet solution for 3 minutes.
14. Rinse in distilled water.
15. Gram's iodine solution for 1 minute.
16. Rinse in distilled water. Blot, but leave moist.
17. Differentiate in a solution of equal parts of aniline and xylene, several changes until section appears light purplish red.
18. Xylene, two changes.
19. Mount with Permount or Histoclad.

RESULTS

Grani-positive organisms -blue
Grani-negativo organisms -red
Other elements - various shades of red to purple

REFERENCE. MacCallum, W. G.: *JAMA 72:193, 1919.*

TAYLOR'S METHOD FOR GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

FIXATION. 10% buffered neutral formalin. TECHNIQUE.
Cut paraffin sections at 6 microns.

SOLUTIONS

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

1% ACID ALCOHOL SOLUTION

(See page 38)

HUCKER'S SOLUTION

Gentian violet, 10% alcoholic	2.0 ml
Distilled water	18.0 ml
Ammonium oxalate, 1% aqueous	80.0 ml

Mix and filter before use.

BASIC FUCHSIN SOLUTION (STOCK)

Basic fuchsin	0.1 gm
Methyl alcohol	95.0 ml
Distilled water	5.0 ml

BASIC FUCHSIN SOLUTION (WORKING)

Basic fuchsin (stock) ..... 5.0 ml
Distilled water ..... 60.0 ml

ETHYL ETHER-ACETONE SOLUTION

(See page 222)

0.1% PICRIC ACID-ACETONE SOLUTION

(See page 223)

ACETONE-XYLENE SOLUTION

Acetone .....
Xylene .....

ACETONE-XYLENE SOLUTION II

Acetone ..... 1 part
Xylene ..... 3 parts

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Harris' hematoxylin solution for 5 -10 minutes.
3. Wash in running water for 1 minute.
4. Differentiate in acid alcohol solution.
5. Wash in running water for 3 minutes.
6. Wash in saturated lithium carbonate solution to intensify blue. Froco this point, carry only one slide at a time.
7. Wash in running water for 5 minutes.
8. Hucker's solution for 2 minutes.
9. Wash quickly in water.
10. Mordam in Gram's iodine solution for 1 minute.
11. Wash in water. Blot, but do not allow to dry, using filter paper moistened with water.
12. Decolorize in ethyl ether-acetone until no more blue color comes off. Blot, but do not allow to dry, using filter paper moistened with ethyl ether-acetone.
13. Working basic fuchsin solution for 3 minutes.
14. Wash in water. Blot, but do not allow to dry, using filter paper moistened with water.
15. Dip in acetone until section begins to decolorize.
16. Pass quickly to picric acid-acetone to decolorize and differentiate until section becomes reddish brown-yellow, approximately 15 seconds.
17. Pass quickly through acetone and xylene solution I, then acetone and xylene solution 11.
18. Clear in xylene, two changes.
19. Mount with Permount or Histoclad.

RESULTS

Gram-positive orgánisms - blue to blue-black
Gram-negative organisms - bright red
Nuclei - brownish red
Erythrocytes - red to greenish yellow
Necrotic tissue - yel lowish green
Cytoplasm -yellow
Connective tissue - red

REFERENCE. Taylor, R. D.: *Amer. J. Clin. Path. 46:472 -474, 1966.* Copyright by Williams and Wilkins Co.

GRIDLEY'S METHOD FOR ENDAMOEBA HISTOLYTICA

FIXATION. 10% buffered neutral. formalin.

TECHNIQUE. Cut paraffin sections at *6* microns.

SOLUTIONS

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(S e e p a g e 3 5)

ANILINE-EOSIN SOLUTION

Eosin Y ..... 1.5 gm
Alcohol, 80% ..... 100.0 MI.
Aniline ..... 3.0 MI.
Glacial acetic acid ..... 1.0 MI.

NAPHTHOL GREEN B SOLUTION

Naphthol. green B ..... 1.0 gm
Distilled water ..... 100.0 MI.
Glacial acetic acid ..... 1.0 ml

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Harris' hematoxylin solution for 10 minutes or Weigert's irou hematoxylin solution for 3 minutes.
3. Wash in running water for 5 minutes.
5. Differentiate in 1% acid alcohol solution, see page 38.
6. Wash in running water for 5 minutes.
7. Blue in ammonia water.
8. Wash in running water for 5 minutes
9. Aniline-eosin solution for 5 minutes.
9. Rinse well in distilled water. sections should be a deep rose color.
10. Naphthol green B solution for 5 minutes.

c 11. Differentiate in 95% alcohol, two changes, until erythrocytes in section are bright rose. Check under microscope.

13. Dehydrate in absolute alcohol, and clear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Amoebae	- blue-green
Nuclei of amoebae	deeper blue-green
Ingested erythrocytes	- deep rose
Connective tissue	- green

REMARKS. This procedure does not stain amoebae differentially but is useful in that it demonstrates the ingested erythrocytes extremely well.

REFERENCE. Gridley, M. *F.: Amer. J. Clin. Path.* 24:243 -244, 1954. Copyright by Williams and Wilkins Co.

GRIDLEY'S METHOD FOR FUNGI

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

4% CHROMIC ACID* SOLUTION

Chromic acid	4.0 gm
Distilled water	100.0 ml

COLEMANS FEULGEN REAGENT

(See page 159)

ALDEHYDE FUCHSIN SOLUTION

(See page 78)

0.25% METANIL YELLOW SOLUTION

(See page 78)

STAINING PROCEDURE. Use control slide.

2. Deparaffinize and hydrate to distilled water.
3. Oxidize in 4% chromic acid for 1 hour.
4. Wash in running water for 5 minutes.
5. Coleman's feulgen reagem for 15 minutes.
6. Wash in running water for 15 minutes.
7. Rinse in 70% alcohol, several changes.
8. Aldehyde fuchsin solution for 30 minutes.
9. Rinse off excess stain with 95% alcohol.
10. Rinse in distilled water.
11. Counterstain lightly with metanil yellow solution for 1 minute.
12. Rinse in distilled water.

*Chromium trioxide

12. Dehydrate **in** 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

13. Mount with Permount or Histoclad.

RESULTS

Mycelia - deep purple
Conidia - deep rose to purple
Background - yellow
Elastic tissue and mucin also, stain deep purple.

REMARKS. An extremely useful technic for fungi. The morphologic detail of yeast forms and hyphae are usually visible, although very old fungi, which were probably nonviable at the time of fixation, are not as well stained as by the Grocott's methenamine-silver technic. The filaments of Nocardia and Actinomyces are *NOT* stained by this method.

Note: Luna, L. G., published "Evaluation of Staining Technic for Pathogenic Fungi." That report can be found in *Amer. J. Med. Techn.* 30:139 -146, 1964.

REFERENCE: Gridley, M. F.: *Amer. J. Clin. Path.* 23:303-307, 1953. Copyright by Williams and Wilkins Co.

GROCOTT'S METHOD FOR FUNGI (GMS)

FIXATION. 10% buffered neutral. formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTION

4% CHROMIC ACID SOLUTION

(See page 229)

5% SILVER NITRATE SOLUTION

(See page 91)

3% METHENAMINE* SOLUTION

(See page 97)

5% BORAX SOLUTION

Borax	5.0 gm
Distilled water	100.0 ml

METHENAMINE-SILVER NITRATE SOLUTION (STOCK)

Silver nitrate, 5 % solution	5.0 ml
Methenamine, 3 % solution	100.0 ml

A white precipitate forms but immediately dissolves on shaking. Clear solution remains usable for months. Store in refrigerator.

[0] Hexamethylenatetramine. Eastman Kodak Co., Rochester, N. Y. 14603

METHENAMINE-SILVER NITRATE SOLUTION (WORKING)

Methenamine-silver nitrate solution (stock) .....25.0 ml
Distilled water .....25.0 ml
Borax, 5/[illegible]solution ..... 2.0 ml
Make fresh.

1% SODIUM BISULFITE SOLUTION

Sodium bisulfite ..... 1.0 gm
Distilled water ..... 100.0 ml

0.1% GOLD CHLORIDE SOLUTION

Gold chloride, 1 [illegible] solution ..... 10.0 ml
Distilled water .....90.0 ml
This solution may be used repeatedly.

2% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 88)

0.2% LIGHT GREEN SOLUTION

(See page 159)

STAINING PROCEDURE. Use control slide.

2. Deparaffinize and hydrate to distilled water.
3. Oxidize in 4% chromic acid solution for 1 hour.
4. Wash in tap water for a few seconds.
5. Sodium bisulfite solution for 1 minute to remove any residual chromic acid.
6. Wash in running water for 5 to 10 minutes.
7. Rinse with three or four changes of distilled water.
8. Place in freshly mixed working methenamine-silver nitrate solution in oven at 58 to 60 ° C for 60 minutes or until section turns yellowish brown.
9. Rinse in six changes of distilled water.
10. Tone in gold chloride solution for 2 to 5 minutes.
11. Rinse in distilled water.
12. Remove unreduced silver with sodium thiosulfate (hypo) solution for 2 to 5 minutes.
13. Wash thoroughly in tap water.
14. Counterstain with working light green solution for 30 to 45 seconds.
15. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
16. Mount with Permount or Histoclad.

RESULTS

Fungi - sharply delineated in black
Mucin - taupe to datk gray
Inner parts of mycelia and hyphae - old rose
Background - pale green

12. Dehydrate in 95/% alcohol, absolute alcohol, and clear in xylene, two changes e each.

13. Mount with Permount or Histoclad.

RESULTS

Mycelia	-deep purple
Conidia	deep rose to purple
Background	yellow

Elastic tissue and mucin also stain deep purple.

REMARKS. An extremely useful technic for fungi. The morphologic detail of yeast forms and hyphae are usually visible, although very old fungi, which were probably nonviable at the time of fixation, are not as well stained as by the Grocott's methenamine-silver technic. The filaments of Nocardia and Actinomyces are *NOT* stained by this method.

Note: Luna, L. G., published "Evaluation of Staining Technic for Pathogenic Fungi." That report can be found in *Amer. J. Med. Techn.* 30:139 -146, 1964.

REFERENCE: Gridley, M. F.: *Amer. J. Clin. Path.* 23:303-307, 1953. Copyright by Williams and Wilkins Co.

GROCOTT'S METHOD FOR FUNGI (GMS)

FIXATION. 10% buffered neutral formalin. TECHNIQUE.

Cut paraffin sections at 6 microns.

SOLUTION

4% CHROMIC ACID SOLUTION

(See page 229)

5% SILVER NITRATE SOLUTION

(See page 91)

3% METHENAMINE* SOLUTION

(See page 97)

5% BORAX SOLUTION

Borax	5.0 gm
Distilled water	100.0 ml

METHENAMINE-SILVER NITRATE SOLUTION (STOCK)

Silver nitrate, 5 1[1-7]o solution	5.0 ml
Methenamine, 3% solution	100.0 ml

A white precipitate forms but immediately dissolves on shaking. Clear solution remains usable for months. Store in refrigerator.

° Hexamethylenatetramine. Eastman Kodak Co., Rochester, N. Y. 14603

METHENAMINE-SILVER NITRATE SOLUTION (WORKING)

Methenamine-silver nitrate solution (stock)	25.0 ml
Distilled water	25.0 ml
Borax, 5/1''/clsolution	2.0 ml

Make fresh.

1% SODIUM BISULFITE SOLUTION

Sodium bisulfite	1.0 gm
Distilled water	100.0 ml

0.1% GOLD CHLORIDE SOLUTION

Gold chloride, 1,0/r, solution	10.0 ml
Distilled water	90.0 ml

This solution may be used repeatedly.

2% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 88)

0.2% LIGHT GREEN SOLUTION

(See page 159)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in 4% chromic acid solution for 1 hour.
3. Wash in tap water for a few seconds.
5. Sodium bisulfite solution for 1 minute to remove any residual chromic acid.
6. Wash in running water for 5 to 10 minutes.
7. Rinse with three or four changes of distilled water.
8. Place in freshly mixed working methenamine-silver nitrate solution in oven at 58 to 60 ° C for 60 minutes or until section turns yellowish brown.
9. Rinse in six changes of distilled water.
10. Tone in gold chloride solution for 2 to 5 minutes.
11. Rinse in distilled water.
12. Remove unreduced silver with Sodium thiosulfate (hypo) solution for 2 to 5 minutes.
13. Wash thoroughly in tap water.
14. Counterstain with working light green solution for 30 to 45 seconds.
15. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
16. Mount with Permount or Histoclad.

RESULTS

Fungi	- sharply delineated in black
Mucin	- taupe to dark gray
Inner parts of mycelia and hyphae	- old rose
Background	- pale green

REMARKS. This technic gives to all forros of fungi, including the filaments of *Actinomyces bovis* and *Nocardia asteroides,* a black-brown coloration. In staining fungi, especially filaments of Nocardia, it has been found that the time of exposure to methenamine-silver nitrate solution for complete development may vary according to type and/or strains suspected. Therefore, if Nocardia is suspected, two slides should be processed, one for 60 minutes and one for 90 minutes, thereby assuring a positive demonstration of any filaments of Nocardia that may exist. Slides previously stained with most other stains may be used by removing cover glasses in xylol and running through alcohols to water. Subsequent chromic acid treatment will remove any remaining stain.

REFERENCE. Grocott, R. G.: *Amer. J. Clin. Path.* 25:975-979, 1955. Copyright by Williams and Wilkins Co.

PICKETT'S FLUORESCENCE METHOD FOR FUNGI

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

0.1% ACRIDINE ORANGE SOLUTION

Acridine orange	O.Igm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Weigert's irou hematoxylin solution for 5 minutes.
3. Wash in running water for 4 minutes.
4. Acridine orange solution for 2 minutes.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with XAM.' RESULTS. Pungi fluoresce brightly against a dark background.

REFERENCE. Pickett, J. P., et al: *Amer. J. Clin. Path.* 34:197 -202, 1960. Copyright by Williams and Wilkins Co.

ATTWOOD'S METHOD FOR AMNIOTIC FLUID EMBOLISM

FIXATION. 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

À~ SOLUTIONS

1 % ALCIAN GREEN SOLUTION

Alcian green ..... 1.0 gm
Distilled water ..... 100.0 ml
Glacial acetic acid ..... 2.0 ml

PHLOXINE-CALCIUM CHLORIDE SOLUTION

Phloxine ..... 0.5 gm
Calcium chloride ..... 0.5 gm
Distilled water ..... 100.0 ml

TARTRAZINE-CELLOSOLVE SOLUTION

Tai trazine ..... 2.5 gm
cellosolve (ethylene glycol monoethyl ether) ..... 100.0 ml

SCOTT'S WATER SOLUTION

(See page 84)

0.5% POTASSIUM PERMANGANATE SOLUTION

(See page 87)

0.5% OXALIC ACID SOLUTION

(See page 79)

4h0~

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Alcian green solution for 2 minutes.
3. Rinse in distilled water.
4. Potassium permanganate solution for 2 minutes.
5. Rinse in distilled water.
6. Oxalic acid solution for 2 minutes.
7. Rinse in distilled water.
8. Mayer's hematoxylin solution. for 3 minutes (see page 33)

9 Rinse and blue in Scott's water solution. for 5 minutes.

10. Phloxine solution for 30 minutes.
11. Rinse in distilled water, then in. cellosolve.
12. Differentiate each slide with tartrazine-cellosolve solution, checking until the phloxine is removed froco erythrocytes.
13. Dehydrate in cellosolve, olear in xylene, two changes each.
14. Mount with Permount or Histoclad.

RESULTS

Nuclei	- blue-black
Mucin, ground substance of cartilage, granules of certain mast cells	- green
Squamous epithelium, fíbrín	-red
Erythrocytes, plasma, collegen	- yellow
Inclusion bodies	- red

REMARKS. This method has been used very successfully in our laboratories for the demonstration of inclusion bodies. For this, however, differentiation at. step 12 must be rigidly controlled.

Note: The permanganate-oxalic bleach is not strictly necessary but it assists in the subsequent ditterentiation of the phloxme and tends to remove any background staining by alcian green. Any excess staining with tartrazine can be removed by rinsing in water until the desired intensity is obtained.

REFERENCE. Attwood, H. D.: *1. Path. Bact.* 76:211-215, 1958.

LENDRUM'S METHOD FOR INCLUSION BODIES

FIXATION. 10% buffered neutral formalin or Zenker's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

0.5% PHLOXINE SOLUTION

Phloxine	1.0 gm
Alcohol, 70%	200.0 ml
Calcium chloride	1.0 grn

2.5% TARTRAZINE SOLUTION

(See page 233)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mayer's hematoxylin solution for 5 -10 minutes.
3. Blue in running water for 15 minutes.
4. Phloxine solution for 30 minutes.
5. Rinse briefly in distilled water and drain slides well.
6. Tartrazine solution until inclusion bodies stand out a bright red on a yellow background.
7. Dehydrate in 95%, alcohol, absoluto alcohol, and clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Inclusion bodies	- red
Nuclei	- blue
Background	- yellow

REFERENCE. Lendrum, A. C.: *J. Path. Bact.* 59:399 -404, 1947.

PAGE-GREEN METHOD FOR INCLUSION BODIES

FIXATION. Any well-fixed tissue.

TECHNIQUE. Cut parattin sections at 6 microns.

4 SOLUTIONS

1% ACID ALCOHOL SOLUTION

(See page 38)

SHORR'S STAINING SOLUTION

Alcohol, 50%	100.0 ml
Biebrich scarlet	1.0 gm
Orange G	0.25 gm
Fast green, FCF	0.075 gm
Phosphotungstic acid	0.5 gm
Phosphomolybdic acid	0.5 gm
Glacial acetic acid	2.0 ml

HARRIS' HEMATOXYLIN SOLUTION

(See page 34)

AMMONIA WATER SOLUTION

(See page 38)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Harris' hematoxylin solution for 3 to 5 minutes.
3. Rinse in water.
4. Differentiate in acid .alcohol solution until there is no hematoxylin in the cytoplasm of the cells. *Check with microscope.*
5. Wash in water.
6. Blue sections in ammonia water solution. *Check with microscope.*
7. Wash in running water for 10 minutes.
8. Shorr's staining solution for 1 minute.
9. Rinse in 95% alcohol. *Check with microscope.* Connective tissue will be a clear light green when differentiation is complete.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

RESULTS

Inclusion bodies	- brilliant red
Connective tissue	- light green
Elastic tissue	- purplish red
Muscle	- red
Keratin	-orange
Erythrocytes	-orange red
Nuclei	- blue

REFERENCE. Page, W. G., and Green, R. G.: *Cornell Vet.* 32:265 -268, 1942.

GIEMSA METHOD FOR RICKETTSÈA

FIXATION. 10% buffered neutral formalin or Zenker's.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

BUFFERED WATER SOLUTION pH 6.8

Buffer salts (pH 6.8)	1 Ogm
Distilled water	1000.0 ml

GIEMSA SOLUTION (STOCK)

Giemsa powder	1.0 **gm**
Glycerin, reagent grade	66.0 ml
Methyl alcohol, acetone free, reagent grade	66.0 ml

See page 127 (Price's Giemsa) for additional information on compounding of Giemsa solution.

GIEMSA SOLUTION (WORKING)

Giemsa solution (stock)	1.0 ml
Buffered water, pH 6.8	50.0 ml

0.2% GLACIAL ACETIC ACID SOLUTION

Glacial acetic acid	0.2 ml
Distilled water	99.8 ml

ROSIN ALCOHOL SOLUTIONS

(See page 119)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in buffer water solution for 60 minutes.
3. Working Giemsa solution overnight.
4. Rinse in buffer water solution.
5. 0.2% glacial acetic acid solution for 1 minute.
6. Rinse in buffer water solution.
7. Differentiate sections individually in working rosin alcohol solution. Check with microscope frequently until rickettsiae appear as violet colored granules (may take up to 3 minutes).
8. Dehydrate in absoluto alcohol and clear in xylene, three changes each.
9. Mount with Permount or Histoclad.

RESULTS

Rickettsia	- violet
Nuclei	- blue
Cytoplasm, connective tissue	pink
Erythrocytes	salmon

REMARKS. The differentiation of step 7 is the most criticai and only if this is properly done can one expect good results. Use the microscope as suggested above for this purpose.

REFERENCE. Histopathology Laboratories, Armed Forces Instituto of Pathology, Washington, D.C. 20305.

PINKERTON'S METHOD FOR RICKETTSIA

FIXATION. Zenker's, Regaud's, or 10% buffered neutral formalin.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% METHYLENE BLUE SOLUTION

Methylene blue	1.0 gm
Distilled water	100.0 ml

0.25% BASIC FUCHSIN SOLUTION

Basic fuchsin	0.25 gm
Distilled water	100.0 ml

0.5% CITRIC ACID SOLUTION

Citric acid	0.5 gm
Distilled water	100.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and olear with sodium thiosulfate (soe pago 41).
3. Methylene blue solution overnight.
4. Rinse in 95% alcohol for 5 seconds or blue color will be lost.
5. Rinse quickly in distilled water for 2 - 3 seconds.
6. Basic fuchsin solution for 30 minutes.
7. Decolorize rapidly in citric acid solution for 1-2 seconds, never more than 3 seconds.
8. Continue differentiation in absoluto alcohol until nuclei stand out blue and rickettsia clumps red.
9. Clear with xylene, two changes.
10. Mount with Permount or Histoclad.

RESULTS

Rickettsia	- bright red
Nuclei	- blue

REMARKS. This is by far the most reliable method available for demonstrating rickettsia in paraffin sections. Good differentiation can be controlled since only erythrocytes and rickettsia must be red in well stained sections. All other structures are light blue.

REFERENCE. Simmons, J. S., and Gentzkow, C. J.: *Laboratory Methods of The United States Army*, 5th ed. Philadelphia, Penna., Lea and Febiger, 1944, p. 572.

LEVADITI-MANOVELIAN METHOD FOR SPIROCHETES

FIXATION. 10% buffered neutral formalin. Specimen should be 1 mm thick. TECHNIQUE. Embed in paraffin after staining is completed (soe Staining Procedure Step 10).

SOLUTIONS

3% SILVER NITRATE SOLUTION

Silvar nitrate	3 0 gm
Distilled water	100.0 ml

REDUCING SOLUTION

Pyrogallic acid	4 0 gm
Formalin, 37 — 40%	5.0 ml
Distilled water	100.0 ml

STAINING PROCEDURE

1. Rinse specimen in tap water, after fixation.
2. Let stand in 95%, alcohol for 24 hours.
S. Transfer to distilled water and leave until the tissue sinks to the bottom, of the contamer.
4. Placo in freslily prepared silvar nitrate solution and keep in 37 °C in the dark for 3 to 5 days, changing the solution three times.
5. Rinse in distilled water.
6. Reducing solution at room temperatura, in the dark for 24 to 72 hours.
7. Rinse in distilled water.
S. Dehydrate in 80% alcohol, 95% alcohol, and absoluto alcohol, two changes, 30 minutes each.
10. Clear in oil of cedarwood for two changes, 1 hour each and infiltrate with two changes of paraffin 45 minutes each.
11. Embed in paraffin.
12. Cut sections at 5 microns and mount on slides.
13. When dry, deparaffinize with xylene, three changes.
14. Mount with Permount or Histoclad.

RESULTS

Spirochetes	- black
Background	- yellow to light brown

REFERENCE. Mallory, F. B.: *Pathological Technique,* New York, Hafner Publishing Co., 1961, p. 293.

WARTHIN -STARRY METHOD FOR SPIROCHETES AND DONOVAN BODIES

FIXATION. 10% buffered neutral formalin. *Avoid chromate fixatives.*

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS. Use chemically clean glassware.

ACIDULATED WATER

Triple distilled water ..... 1000.0 ml

Add enough 1% aqueous citric acid to bring water to pH 4.0.

1% SILVER NITRATE SOLUTION **(For impregnation)**

Silver nitrate, C.P. crystals ..... 1.0 gm
Acidulated water ..... 100.0 ml

2% SILVER NITRATE SOLUTION (For developer)

Silver nitrate, C.P. crystals ..... 2.0 gm
Acidulated water ..... 100.0 ml

5% GELATIN SOLUTION

Sheet gelatin, high grade ..... 10.0 gm
Acidulated water ..... 200.0 ml

0.15% HYDROQUINONE SOLUTION

Hydroquinone, crystals, photographic quality ..... 0.15 gm
Acidulated water ..... 100.0 ml

Keep 2% silver nitrate, 5% gelatin, and 0.15% hydroquinone in 50 ml Erlenmeyer flasks, in a flotation bath at 54 °C until developer is made.

DEVELOPER SOLUTION

Silver nitrate solution, 2% 1 5 m1
Gelatin solution, 5% ..... 3.75 ml
Hydroquinone solution, 0.15% ..... 2.0 ml

Combine in the order given in small beaker, making certain solutions are mixed well. *Prepare immediately before use.*

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to triple distilled water.
2. Impregnate with silver nitrate solution heated in a flotation bath to 43 °C for 30 minutes. Prepare the developer solution at this point. (See *Note)*
3. Flood sections, that have been, laid across glass rods, with the developer solution which *must be used as soou as it is mixed.* Allow sections to develop until they are light brown or yellow. Check known control under the microscope. The spirochetes should be black and the background light brown or yellow. (See *Note)*
4. Wash quickly and thoroughly in hot tap water, approximately 56 °C.
5. Rinse in distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
7. Mount with Permount or Histoclad.

RESULTS

Spirochetes, Donovan bodies	- black
Background	- pale yellow to light brown

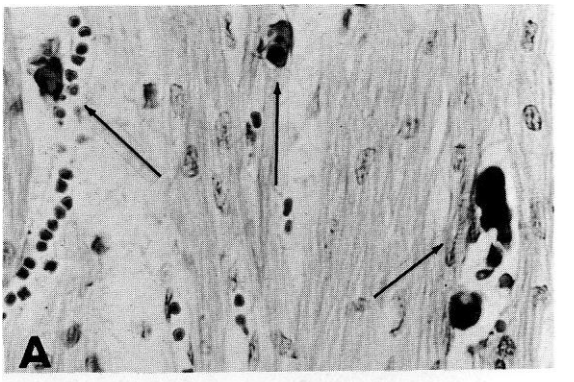
REMARKS. It may be necessary to prolong development of sections for the demonstration of Donovan bodies. Certain hematogenous pigments, nuclei and melanin have a greater attraction for silvar than do spirochetes, and it is difficult to stain the spirochetes in Glose proximity to these elements. By lowering the pH of the acidulated solution to 3.6 and prolonging the development, the spirochetes may be demonstrated in the areas of competition; however, the part of the section not containing competitiva elements may be overstained and useless. Sections can be restained to increase the amount of development if microscopic observation of the known positive tissue shows pale spirochetes or nona at all.

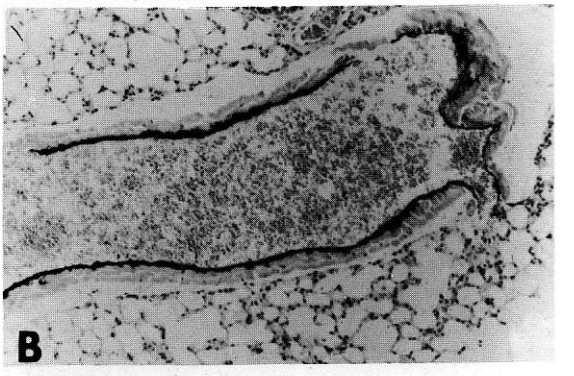
Note. Use paraffin coated forceps, particularly at step 2 and step 3.

REFERENCES. Kerr, D. A.: *Amer. J. Clin. Path. Tech. Suppl.* 8:63 -67, 1938. Copyright by Williams and Wilkins Co. (AFIP modification)

C. H. Bridges, and L. G. Luna studied permissible variations of this technic in the AFIP laboratories. Their report can be found in *Lab. Invest.* July-August, 1957.

#6, PSEUDO CALCIFICATION — DUE TO CALCIUM ACETATE USED' AS BUFFERING AGENT.

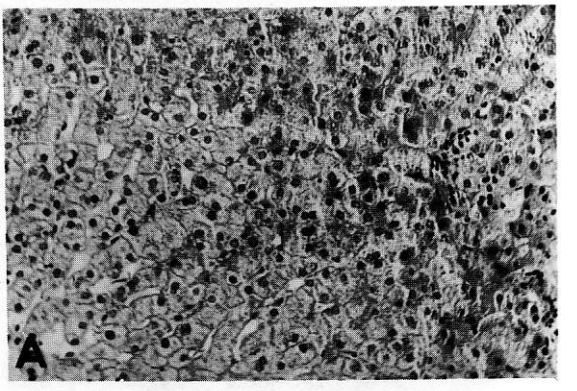


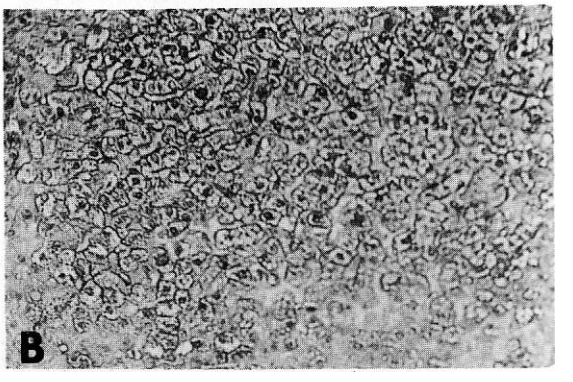


B. Heart muscle (arrow). Note absence of true tissue reaction.

C. Lung. Deposits on intima) layer.

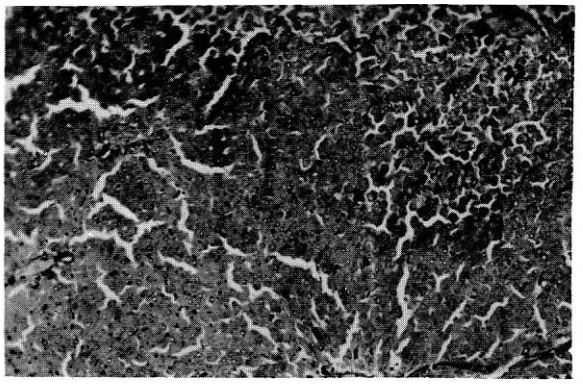
#7. INADEQUATE REMOVAL OF PICRIC ACID AFTER BOUIN'S FIXATION.





A. Section cut and stained 24 hours after fixation.

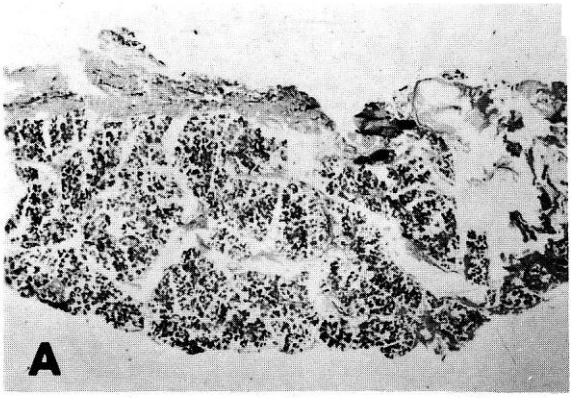
B. Section (froco same block) cut and stained 4 weeks after fixation.
Note destruction of tissue due to action of picric acid.

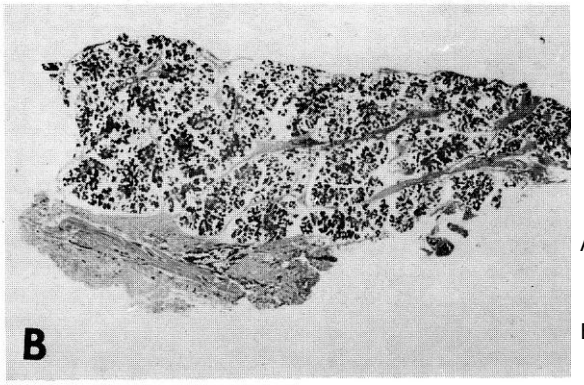


Dry-Earth Effect: Due to inadequate dehydration, clearing and impregnation. This effect takes place in the clearing xylene following H & E Staining.

#11. Improper impregnation during processing: Note the uneven staining and spreading effect.

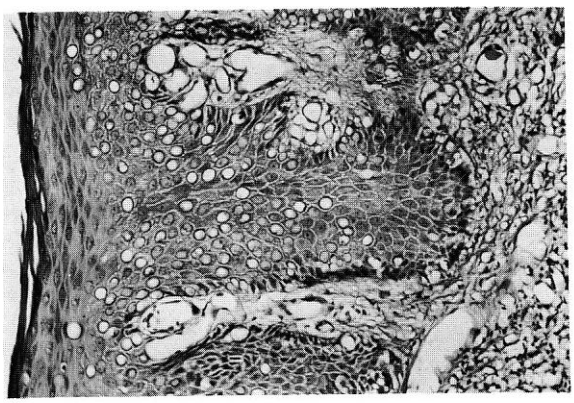
#12. IMPROPER DEHYDRATION, CLEARING AND IMPREGNATION.



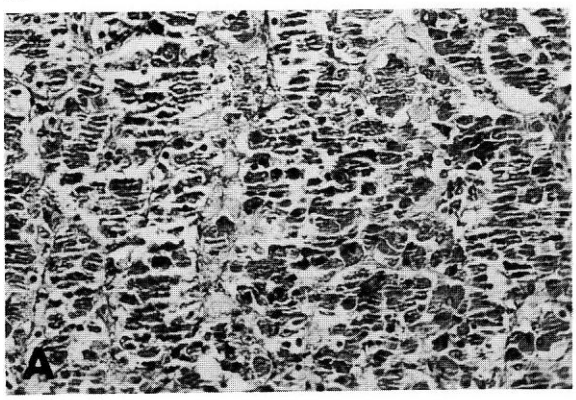


A. Spreading effect created when an improperly processed specimen is placed on tissue floatation bath.

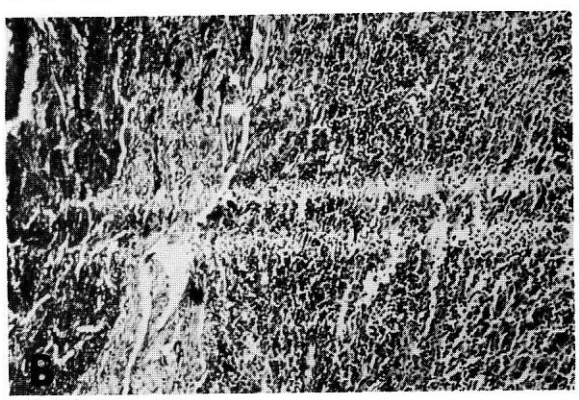
B. Same specimen after reprocessing *(Note:* Lack of spreading).

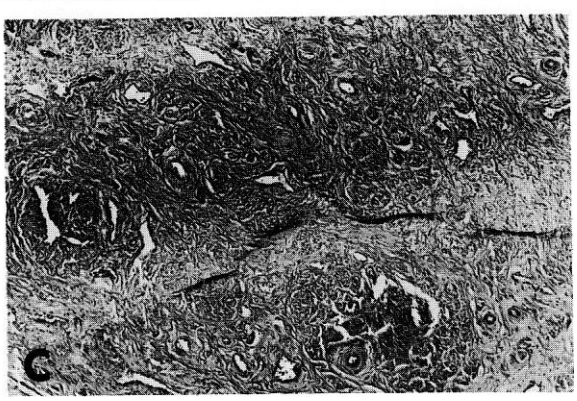


#14. A more dramatic effect is seen on this skin section. Biopsy was taken, put in formalin, then placed in a mail box with the outside temperatura below freezing point. Apparently the low temperaturas caused ice crystals to forro within the cells and the tissues in turn became fixed. Following thawing the vacuoles remained as fixed artifacts.



#15. CUTTING ARTIFACTS.



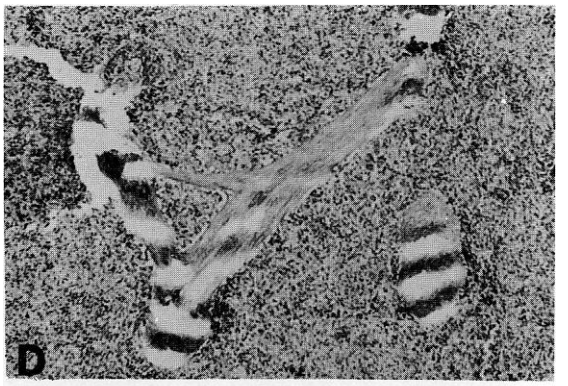


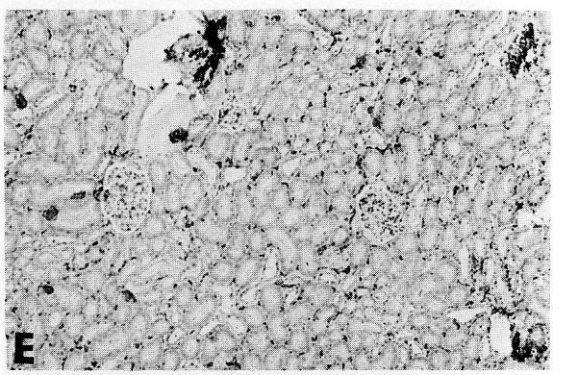
A. Shattering effect due to dull knife. *Note:* It is impossible to recognize this section of a pituitary gland due to the morphologic destruction of the individual cells.

B. Lengthwise scratches may be due to (1) nicks in knife, (2) lint on knife edge, (3) calcified area in the tissue, (4) dirty knife edge, ar (5) exogenous pigments.

C. Venetian blind effect (thick and thin areas) produced when an extremely fibrous specimen is cut with a thin microtome knife.

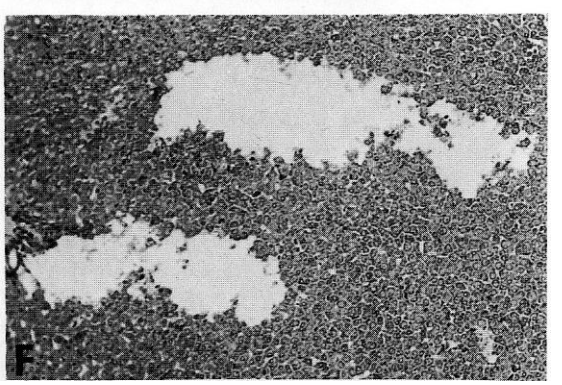
#15. CUTTING ARTIFACTS (continued)



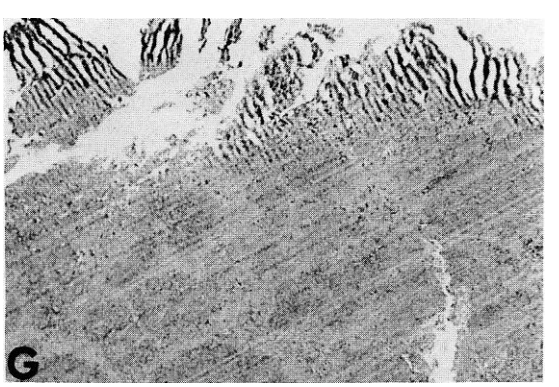


D. Thick and thin areas noted on the collagen of this section are due to microtome knife-holder loose set screw. Note surrounding tissue is not affected indicating knife was sharp.

E. The displacement of blood vessels seen in this section is due to an excessively *escute* knife angle. Note surrounding tissue is not affected indicating knife was sharp.

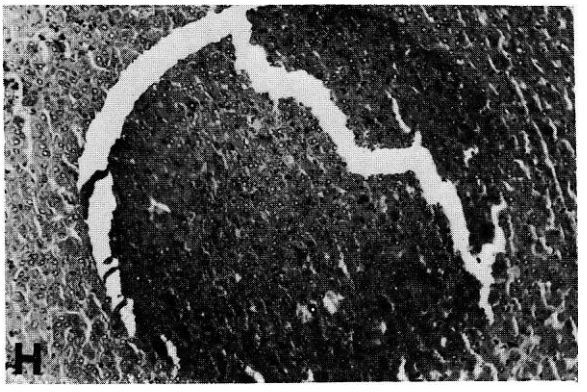


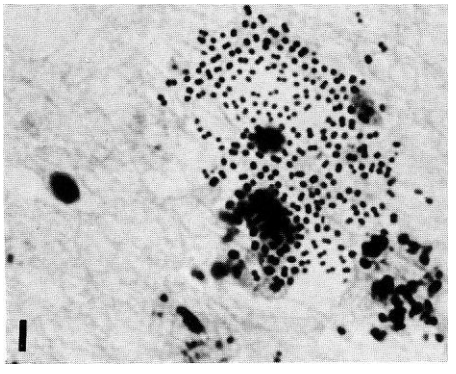
F. Moth-eaten effect is produced when specimens are over-processed causing excessiva dryness. The effect is produced while rough-cutting (facing block) since the dry tissue chies out as the knife traveis through the specimen. This artifact can be eliminated by soaking the block with cotton, see paga 26.



G. Excessiva hardening of peripheral margina due to over-processing. lengthwise scatches are produced when fragmenta of tissue froco the periphery are dragged through the section.

#15. CUTTING ARTIFACTS *(continued)*

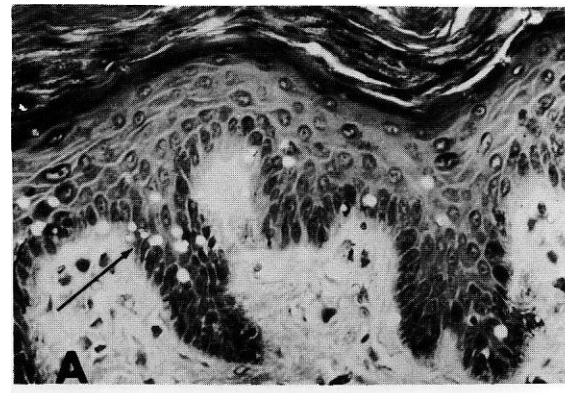


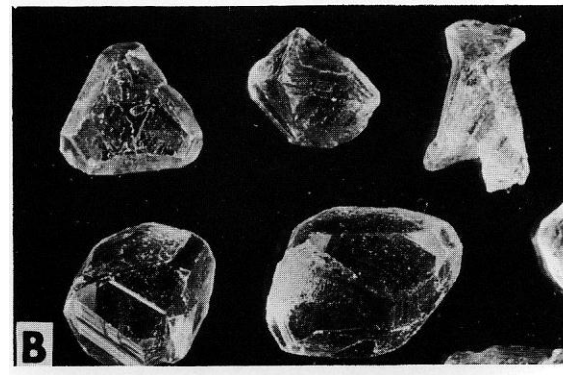


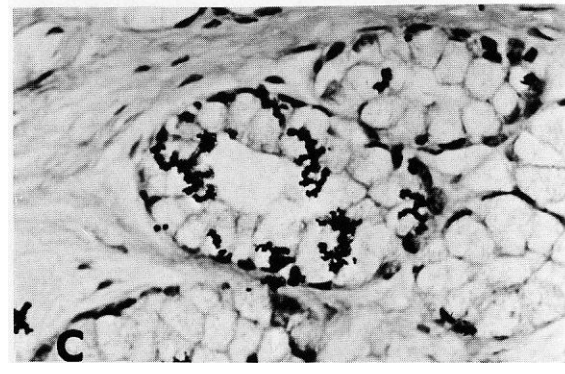
H. Air bubbles produced while laying the ribbon on the floatation bath. These bubbles can be removed by gentle manipulation with a camel-hair brush immediately after sections are picked up on slide.

I.U. Coccoid and rod-shaped type bacteria produced by the use of gelatin in the floatation bath as a section adhesive. Artifact can be prevented by daily careful washing with good detergent of the water both and utensils, such as camel-hair brush and forceps used to tease sections onto slides.

#16. STAINING ARTIFACTS





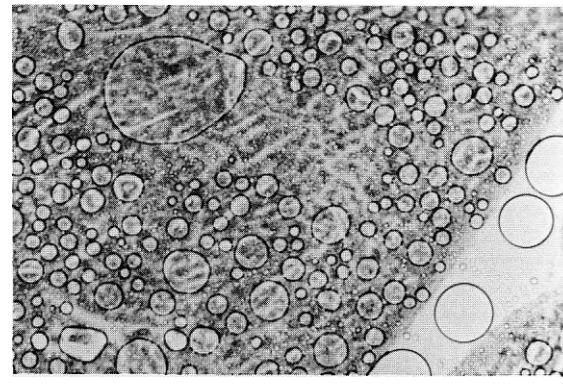


Aluminum ar potassium alum crystals are produced in hematoxylin when ingrediente are improperly compounded *(Note:* The alum must be mixed thoroughly when making hematoxylin).

B. Partially polarized section of skin showing alum crystals (arrow)

C. Alum crystals removed from bottom of container of improperly compounded hematoxylin solution.

D. Hematein crystals resulting from breakdown of hematoxylin containing alum not completely dissolved.



#17. Moisture deposited on finished slide due to water in the absolute alcohol baths.

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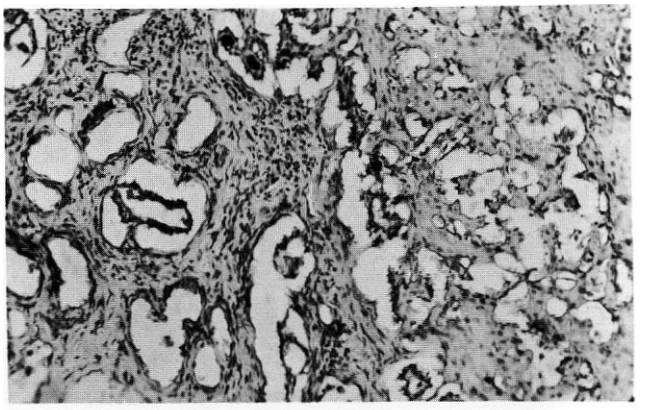
Ezrin Pituitary stain, 113 Woelcke's
Myelin Sheath stain, 206 Wolbach's
Giemsa stain for
bacteria, 119
rickettsia, 119

Z

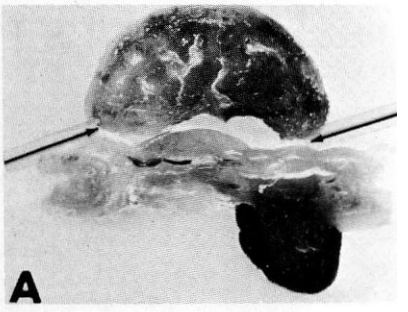
Zenker's fixative, 4

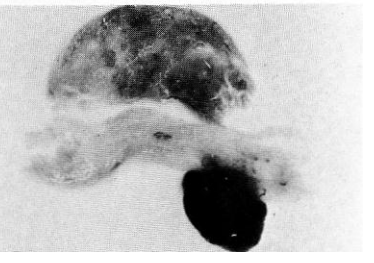
Zenker-formalin (Helly's) fixative, 4
Ziehl-Neelsen Acid Fast stain, 220



#11. Dry homogeneous effect due to exposure to excessive heat during impregnation. *Note:* This can be readily recognized by grayish-yellow coloration of various parts of section.

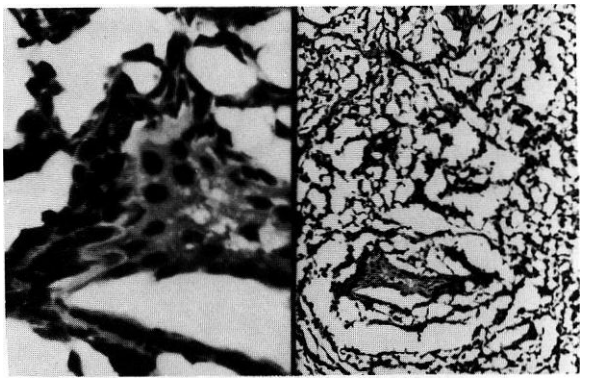
#12. IMPROPER PROCESSING.





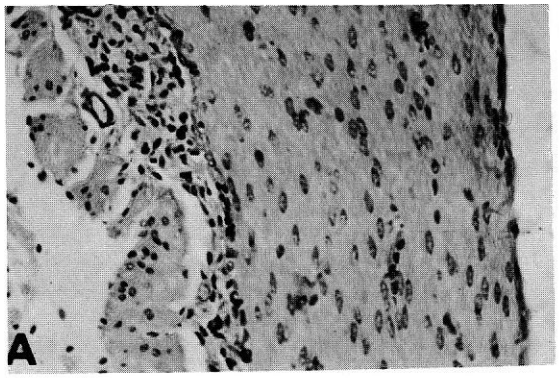
A. Note crevices and hair line separation between tissue and paraffin (arrow). The lack of proper processing of tissue will cause subsequent sectioning problems.

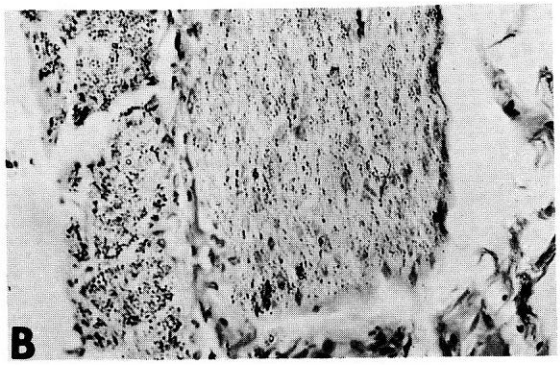
C. Same specimen reprocessed.

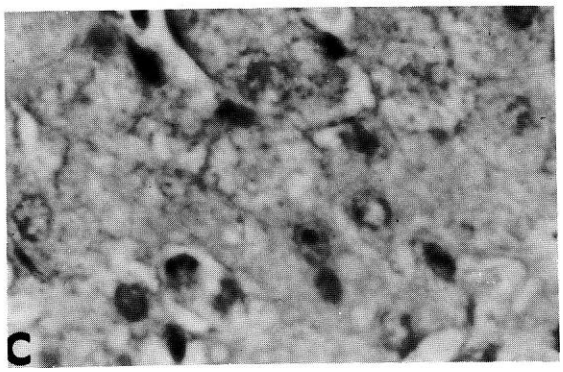


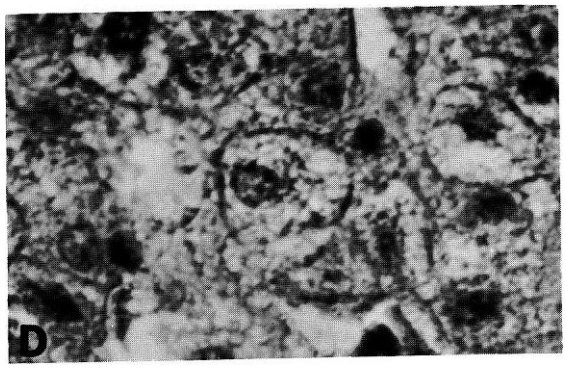
#13. This artifact simulates exposure to excessive heat, however, it is produced by the formation of ice crystals during frozen sectioning and subsequent parafin processing.

#18. COVER SLIPPING ARTIFACTS.









A. Section mounted immediately following removal from xylol.

B. Section allowed to dry bafore mounting.

C. Section mounted with excessiva mounting media giving cloudy result.

D. Section mounted immediately following removal from xylol with correct amount of mounting media.